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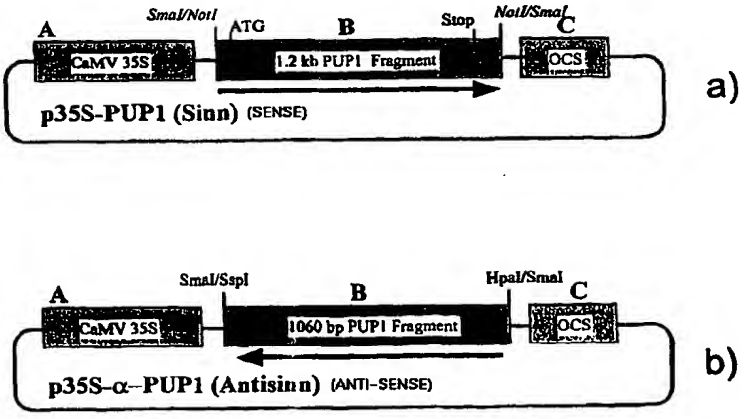
(54) Title: NUCLEIC ACIDS THAT CODE FOR A NUCLEOBASE TRANSPORTER

(54) Bezeichnung: NUKLEINSÄUREN, DIE NUKLEOBASENTRANSPORTER KODIEREN



(57) Abstract

The invention relates to a nucleic acid that codes for a vegetable or animal nucleobase transporter. The invention also relates to the uses of said nucleic acid. The invention further relates to a fragment of nucleic acid, a construct containing said nucleic acid and/or a fragment thereof and a host cell with said nucleic acid, fragment and/or construct. The invention also relates to a method for producing a transgenic plant using the aforementioned nucleic acid and a method for influencing the nucleobase transport properties of a plant, part of a plant, a plant cell and/or seeds.

(57) **Zusammenfassung**

Die Erfindung betrifft eine Nukleinsäure, die für einen pflanzlichen oder tierischen Nukleobasentransporter kodiert, und verschiedene Verwendungen dieser Nukleinsäure. Ferner betrifft die Erfindung ein Fragment der Nukleinsäure, ein Konstrukt, das die Nukleinsäure und/oder ein Fragment davon enthält, und eine Wirtszelle mit der Nukleinsäure, dem Fragment und/oder dem Konstrukt. Von der vorliegenden Erfindung umfasst werden ferner ein Verfahren zum Herstellen einer transgenen Pflanze unter Verwendung der vorgenannten Nukleinsäure sowie ein Verfahren zum Beeinflussen der Nukleobasentransporteigenschaften einer Pflanze, eines Pflanzenteils, einer Pflanzenzelle und/oder von Samen.

Nukleinsäuren, die Nukleobasentransporter kodieren

Die vorliegende Erfindung betrifft eine Nukleinsäure, die für einen pflanzlichen oder tierischen Nukleobasentransporter kodiert, und ihre Verwendung. Ferner betrifft die vorliegende Erfindung ein Fragment der Nukleinsäure, ein Konstrukt, das die Nukleinsäure und/oder ein Fragment davon enthält, und eine Wirtszelle. Von der vorliegenden Erfindung umfaßt werden ferner ein Verfahren zum Herstellen einer transgenen Pflanze sowie ein Verfahren zum Beeinflussen der Nukleobasentransporteigenschaften einer Pflanze, eines Pflanzenteils, einer Pflanzenzelle und/oder von Samen.

Transporter spielen in der Funktion eines Organismus eine besondere Rolle. Zum einen entscheiden sie über die Aufnahme oder Abgabe eines Stoffes in eine oder aus einer Zelle bzw. einem Organismus, andererseits steuern sie den Transport und die Verteilung der Stoffe zwischen den Zellen. Transporter stehen in der Regel am Anfang oder Ende eines Stoffwechselwegs und nehmen daher grundsätzlich übergeordnete Kontrollfunktionen wahr.

Zu den Transportmetaboliten, die als Bausteine für Nukleinsäuren dienen, zählen Purinbasen, Pyrimidinbasen und die daraus abgeleiteten Nukleoside und Nukleotide. Die Aufnahme dieser Substanzen hat zum Beispiel während der Pollenkeimung und der frühen Entwicklung des Embryos in keimenden Samen eine wichtige physiologische Bedeutung bei der Bereitstellung von Vorstufen für die Nukleinsäuresynthese. Cytokinine sind als Phytohormone strukturell eng mit den Purinbasen und den Purinnukleosiden verwandt. Diese Phytohormone regulieren viele Prozesse während der Pflanzenentwicklung. Über den Ursprung dieser Hormone ist zwar wenig bekannt, ihr effektiver Transport in der Pflanze ist jedoch für deren Entwicklung und Funktion von entscheidender Bedeutung.

In Bakterien wurden Nukleobasen-Transportsysteme für Adenin, Cytosin und Uracil charakterisiert und die entsprechenden Gene konnten kloniert werden. In der Bäckerhefe *Saccharomyces cerevisiae* wurden bisher drei unterschiedliche, aktive Transportsysteme für Nukleoside und Nukleobasen sowohl genetisch als auch physiologisch gut charakterisiert. Nukleosidtransportersysteme sind in einer Vielzahl von Säugetierzellen beschrie-

ben und charakterisiert worden. Neben diesen Nukleosidtransportersystemen wurden auch spezifische Transportsysteme für Nukleobasen in Säugetierzellen beschrieben.

In höheren Pflanzen sind Transportprozesse zur Verteilung von Assimilaten, Metaboliten und Phytohormonen von entscheidender physiologischer Bedeutung. Über den Transport von Nukleobasen und deren Derivate in Pflanzen ist jedoch nur sehr wenig bekannt und es wurden bisher im Gegensatz zu Bakterien, Pilzen und Säugetieren nur wenige Transportsysteme für diese Substanzen beschrieben. Eine Aufklärung der Nukleobasen-Transportvorgänge bei Pflanzen, die zu ähnlich detaillierten Informationen wie bei anderen Organismen führen würde, liegt nicht vor und ist wegen der Schwierigkeit der molekularbiologischen Analyse von Mutationen in Pflanzen kaum durchführbar. Es besteht jedoch ein großes Interesse an der Identifizierung und Charakterisierung von Pflanzengenen, die für Nukleobasentransporter bzw. für Transporter für chemisch verwandte Stoffe kodieren. Ferner besteht aufgrund der zentralen Funktion der Transporter ein großes Interesse an Pflanzen, die in der Lage sind, größere Mengen Nukleobasen und ihre Derivate zu transportieren, sowie an der Bereitstellung von Möglichkeiten zur Veränderung der Verteilung von Nukleobasen in transgenen Pflanzen und Mutanten.

Der vorliegenden Erfindung liegt die Aufgabe zugrunde, für pflanzliche oder tierische Nukleobasentransporter kodierende Nukleinsäuren bereitzustellen.

Diese Aufgabe wird erfindungsgemäß durch eine Nukleinsäure gelöst, ausgewählt aus:

a) Nukleinsäure, die erhältlich ist durch Komplementieren Nukleobasentransporter-defizienter Wirtszellen mit einer pflanzlichen oder tierischen Genbank und Selektieren auf Nukleobasentransporter-positive Wirtszellen;

b) Nukleinsäure mit einer Sequenz, die für ein Protein mit einer Sequenz nach SEQ ID NO 8 oder SEQ ID NO 9 kodiert;

c) Nukleinsäure, die mit einer Nukleinsäure nach b) hybridisiert;

d) Nukleinsäure, die unter Berücksichtigung der Degeneration des genetischen Codes mit einer Nukleinsäure nach b) oder mit der zu b) komplementären Sequenz hybridisieren würde;

e) durch Substitution, Addition, Inversion und/oder Deletion einer oder mehrerer Basen erhaltener Derivate einer Nukleinsäure nach a) bis d);

f) Komplementäre Nukleinsäure zu einer Nukleinsäure nach einer der Gruppen a) bis e);

ausgenommen sind Nukleinsäuren mit einer Sequenz nach einer der SEQ ID NO 3 bis 5.

Der Begriff "Nukleobase", wie hier verwendet, umfaßt nicht nur Nukleobasen und ihre Derivate, sondern auch mit den Nukleobasen chemisch verwandte Stoffe, beispielsweise Adenin, Guanin und seine Derivate, wie Xanthin, Hypoxanthin, Allantoin, Allantoat, Urat, Xanthosin oder Inosin, Cytosin und seine Vorstufen und Derivate wie Barbiturate oder Folsäure, Cytokinine, wie z.B. Zeatin, Isopentenyladenin oder Kinetin, und bestimmte Alkaloide, wie z.B. Koffein, Theobromin oder Nikotin. Diese pflanzlichen Alkaloide zeigen eine große strukturelle Ähnlichkeit mit der Nukleobase Purin, da sie ebenfalls basische, N-haltige Heterocyclen aufweisen. Cytokinine enthalten Adenin als hydrophiles Grundgerüst, an dessen Aminogruppe in der Position 6 eine unpolare Seitenkette von relativ geringer Spezifizität sitzt. Bei dem Kinetin handelt es sich um ein künstliches Cytokinin, welches vermutlich gar nicht natürlich in der Pflanze vorkommt. Weiterhin können die Nukleobasen modifiziert sein und an Zucker oder andere Bausteine gekoppelt sein, z.B. Adenosin als Ribosid von Adenin, Cytidin als Ribosid von Cytosin, oder Cytokininriboside.

Der Ausdruck "Nukleobasentransporter" im Sinne der Erfindung bezeichnet ein Protein, das an dem Transport von mindestens einem der vorgenannten Metabolite durch eine Biomembran beteiligt ist. Dieser Transport kann aktiv oder passiv erfolgen. Zum Nachweis der Nukleotransporter-Aktivität kann beispielsweise die von Ninnemann et al. (1994, *EMBO J.* 15, 3464-3471) beschriebene Methode angewandt werden.

"Komplementation", wie hier verwendet, bezeichnet eine sich im Phänotyp widerspiegelnde Kompensation eines genetischen Funktionsdefektes unter Beibehaltung der dem

Defekt zugrundeliegenden Mutationen. Komplementation im Sinne der Erfindung liegt beispielsweise vor, wenn ein genetischer Defekt in einem Gen (z.B. das FCY2-Gen in *Saccharomyces cerevisiae*) durch die Anwesenheit eines gleichartigen, intakten Gens (z.B. das PUP1-Gen aus *Arabidopsis thaliana*) aufgehoben wird, das die Funktion des defekten Gens übernimmt.

Unter "Nukleobasentransporter-defizienten Wirtszellen" im Sinne der Erfindung versteht man Zellen, die aufgrund eines genetischen Defektes negativ veränderte Nukleobasentransporteigenschaften aufweisen, die zu einem negativ selektierbaren Phänotyp führen. Zur Ausführung der Erfindung bevorzugteNukleobasentransporter-defiziente Wirtszellen sind eukaryontische Zellen, z.B. pflanzliche oder tierische Zellen. Nukleobasentransporter-defiziente Hefezellen sind besonders bevorzugt.

Nukleobasentransporter-positive Wirtszellen enthalten eine Nukleinsäure, die zumindest zur partiellen Aufhebung des genetischen Defekts führt, und zeigen daher einen positiv selektierbaren Phänotyp.

Die Identifikation eines Nukleobasentransporters kann beispielsweise durch Komplementation von spezifischen Mutationen in der Hefe *Saccharomyces cerevisiae* erfolgen. Zur Isolierung eines Gens, das ein Transportermolekül kodiert, aus einer pflanzlichen oder tierischen Genbank müssen zunächst geeignete Hefe-Mutanten verfügbar sein, die aufgrund eines Defektes in diesem Transportermolekül nicht in der Lage sind, eine bestimmte Substanz aufzunehmen. Eine Mutante, die nicht in der Lage ist, in Medien mit Nukleobasen als einziger Stickstoffquelle zu wachsen, ist beispielsweise die von Grenson (Grenson, 1969, *Eur. J. Biochem.* 11, 249-260; Polak & Grenson, 1973, *Eur. J. Biochem.* 32, 276-282) beschriebene Mutante *fcy2* (Stamm MG887). Um eine Komplementation mit pflanzlichen oder tierischen Genen durchführen zu können, wurde zusätzlich das URA3-Gen zerstört und somit eine Uracil-Auxotrophie erzeugt (MG887ura3$^-$).

Zum Erhalten einer erfindungsgemäßen Nukleinsäure kann eine geeignete Hefemutante, wie beispielsweise die *fcy2/ura3*-Mutante, mit für die Verwendung in Hefe geeigneten Expressions-Plasmiden, die als Insertion cDNA-Fragmente aus einer pflanzlichen oder

tierischen cDNA-Bibliothek tragen, transformiert werden. Durch Selektion von Transformanten, die infolge der Expression pflanzlicher oder tierischer cDNA-Sequenzen in der Lage sind, auf Nukleobasen als einziger Stickstoffquelle zu wachsen, werden pflanzliche bzw. tierischen Nukleobasentransporter identifiziert.

Es wurde nun überraschend gefunden, daß bei Expression einer cDNA-Bibliothek, beispielsweise aus Keimlingsgewebe von A*rabidopsis thaliana*, mittels für die Verwendung in Hefe geeigneter Expressions-Plasmide, die den Promotor der Phosphoglyceratkinase aus Hefe enthalten, die Komplementation der Mutation *fcy2* möglich ist, wenn die Expressions-Plasmide bestimmte pflanzliche cDNA-Fragmente enthalten. Diese cDNA-Fragmente kodieren pflanzliche Nukleobasentransporter und werden von der vorliegenden Erfindung umfaßt.

Das Merkmal "Nukleinsäure, die mit einer Nukleinsäure nach b) hybridisiert", wie hier verwendet, weist auf eine Nukleinsäure hin, die unter mäßig stringenten Bedingungen mit der Nukleinsäure nach b) hybridisiert. Beispielsweise kann die Hybridisierung mit der radioaktiven Genprobe in einer Hybridisierungslösung (25% Formamid; 5 x SSPE; 0,1% SDS; 5 x Denhardt-Lösung; 50 µg/ml Heringsperma-DNA; zur Zusammensetzung der Einzelkomponenten vgl. Sambrook et al., 1989, Molecular Cloning: A laboratory manual, 2. Aufl., Cold Spring Harbor Laboratory Press, NY, USA) 20 Stunden lang bei 37°C erfolgen. Die anschließende Entfernung unspezifisch gebundener Probe kann durch mehrfaches Waschen der Filter in 2 x SSC/ 0,1% SDS bei 42°C erfolgen. Vorzugsweise werden die Filter mit 0,5 x SSC / 0,1% SDS, besonders bevorzugt mit 0,1 x SSC / 0,1% SDS bei 42°C, gewaschen.

Die erfindungsgemäßen Nukleinsäuren können in Plasmide eingebracht und mittels Standardverfahren der Mikrobiologie einer Mutagenese oder einer Sequenzveränderung durch Rekombination unterzogen werden. Dadurch ist eine besonders einfache Veränderung der Spezifität des Nukleobasentransporters möglich. Nukleinsäuren, die für veränderte Nukleobasentransporter kodieren, können beispielsweise zur Transformation von landwirtschaftlich genutzten Pflanzen verwendet werden, mit dem Ziel, transgene Pflanzen herzustellen. Mit Hilfe von Standardverfahren (vgl. Sambrook et al., 1989, Molecular cloning: A laboratory manual, 2. Aufl., Cold Spring Harbor Laboratory Press, NY,

USA) können Basenaustausche vorgenommen oder natürliche oder synthetische Sequenzen hinzugefügt werden. Für die Verbindung von Fragmenten untereinander können an die Fragmente Adaptoren oder "Linker" angefügt werden. Ferner können Manipulationen, die passende Restriktionsschnittstellen bereitstellen oder überflüssige Sequenzen oder Restriktionsschnittstellen entfernen, eingesetzt werden. Um Insertionen, Deletionen oder Substitutionen, wie z.B. Transitionen und Transversionen, vorzunehmen, bedient man sich bekannter Methoden, wie z.B. der *in vitro*-Mutagenese, "primer repair", Restriktion oder Ligation. Zur Analyse der erfindungsgemäßen Nukleinsäure werden allgemeine Methoden, wie z.B. Sequenz- oder Restriktionsanalyse, sowie weitere biochemisch-molekularbiologische Methoden verwendet.

Eine Nukleinsäure, die ein Polypeptid oder Protein mit Nukleobasentransporter-Aktivität kodiert, das über seine gesamte Sequenz mindestens 40%, vorzugsweise mindestens 60%, besonders bevorzugt mindestens 80% Homologie zu einem von der Nukleinsäure gemäß SEQ ID NO 1 oder der Nukleinsäure gemäß SEQ ID NO 10 kodierten Polypeptid aufweist, wird von der vorliegenden Erfindung ebenfalls umfaßt.

Im Sinne der Erfindung bezieht sich der Ausdruck "mindestens 40%, vorzugsweise mindestens 60%, besonders bevorzugt mindestens 80% Homologie" auf Übereinstimmung auf der Ebene der Aminosäuresequenz, die gemäß bekannter Verfahren, z.B. der computergestützten Sequenzvergleiche (Basic local alignment search tool, S.F. Altschul *et al.*, J. Mol. Biol. 215 (1990), 403-410) bestimmt werden kann.

Der dem Fachmann bekannte Ausdruck „Homologie" bezeichnet den Grad der Verwandtschaft zwischen zwei oder mehrerer Polypeptid-Molekülen, der durch die Übereinstimmung zwischen den Sequenzen bestimmt wird, wobei unter Übereinstimmung sowohl identische Übereinstimmung als auch konservativer Aminosäure-Austausch zu verstehen ist. Der Prozentsatz der „Homologie" ergibt sich aus dem Prozentsatz übereinstimmender Bereiche in zwei oder mehr Sequenzen unter Berücksichtigung von Lücken oder anderen Sequenzbesonderheiten.

Der Begriff "konservativer Aminosäure-Austausch" bezieht sich auf einen Austausch eines Aminosäurerestes durch einen anderen Aminosäurerest, wobei der Austausch

nicht zu einer Änderung der Polarität oder Ladung führt. Ein Beispiel für einen konservativen Aminosäure-Austausch ist der Austausch eines unpolaren Aminosäurerestes durch einen anderen unpolaren Aminosäurerest. Konservative Aminosäure-Austausche im Sinne dieser Erfindung sind: G = A = S, I = V = L = M, D = E, N = Q, K = R, Y = F, S = T, G = A =I = V = L = M = Y = F = W = P = S = T.

Die Homologie miteinander verwandter Polypeptid-Moleküle kann mit Hilfe bekannter Verfahren bestimmt werden. In der Regel werden spezielle Computerprogramme mit den besonderen Anforderungen Rechnung tragenden Algorithmen eingesetzt. Bevorzugte Verfahren zur Bestimmung der Homologie erzeugen zunächst die größte Übereinstimmung zwischen den untersuchten Sequenzen. Computerprogramme zur Bestimmung der Homologie zwischen zwei Sequenzen umfassen, sind jedoch nicht eingeschränkt auf, das GCG-Programmpaket, einschließlich GAP (Devereux, J., *et al.*, Nucleic Acids Research 12 (12): 387 (1984); Genetics Computer Group University of Wisconsin, Madison, (WI)); BLASTP, BLASTN und FASTA (Altschul, S. *et al.*, J. Molec Biol 215:403/410 (1990)). Das BLAST X Programm kann vom National Centre for Biotechnology Information (NCBI) und aus weiteren Quellen bezogen werden (BLAST Handbuch, Altschul S., *et al.*, NCB NLM NIH Bethesda MD 20894; Altschul, S., *et al.*, J. Mol. 215:403/410 (1990)). Auch der bekannte Smith Waterman-Algorithmus kann zur Bestimmung von Homologie verwendet werden.

Bevorzugte Parameter für den Sequenz-Vergleich umfassen die nachstehenden:

Algorithmus:	Needleman und Wunsch, J. Mol. Biol 48:443-453 (1970)
Vergleichsmatrix:	BLOSUM 62 von Henikoff und Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919 (1992)
Lücken-Wert (Gap Penalty):	12
Lückenlängen-Wert (Gap Length Penalty):	4
Schwellenwert der Ähnlichkeit:	0

Das GAP-Programm ist auch zur Verwendung mit den vorstehenden Parametern geeignet. Die vorstehenden Parameter sind die Standardparameter (default parameters) für Aminosäuresequenz-Vergleiche.

Weitere beispielhafte Algorithmen, Lücken-Öffnungs-Werte (gap opening penalties), Lückenausdehnungs-Werte (gap extension penalties), Vergleichsmatrizen einschließlich der im Programm-Handbuch, Wisconsin-Paket, Version 9, September 1997, genannten können verwendet werden. Die Auswahl wird von dem durchzuführenden Vergleich abhängen und weiterhin davon, ob der Vergleich zwischen Sequenzpaaren, wobei GAP oder Best Fit bevorzugt sind, oder zwischen einer Sequenz und einer umfangreichen Sequenz-Datenbank, wobei FASTA oder BLAST bevorzugt sind, durchgeführt wird.

Von der Erfindung wird ebenfalls eine Nukleinsäure umfaßt, die ein Polypeptid oder Protein mit Nukleobasentransporter-Aktivität kodiert, das über einen Teilbereich von mindestens 20 Aminosäuren mindestens 60%, vorzugsweise mindestens 75%, besonders bevorzugt mindestens 90% Homologie zu einem der von der Nukleinsäure gemäß SEQ ID NO 1 und der Nukleinsäure gemäß SEQ ID NO 10 kodierten Polypeptide aufweist. Vorzugsweise kodiert die erfindungsgemäße Nukleinsäuresequenz ein Polypeptid oder Protein mit Nukleobasentransporter-Aktivität, das eine Aminosäuresequenz umfaßt, die mindestens 70%, vorzugsweise mindestens 80%, besonders bevorzugt mindestens 90% Homologie zu einer der folgenden Aminosäuresequenzen aufweist:

(a) L Y A X G L X Y L P V S T X S L I X X X Q L A F X A X F S (SEQ ID NO 11)

wobei X an den Positionen 4 und 7 für eine hydrophobe Aminosäure (G, A, I, V, L, M, Y, F, W, P, S oder T), an den Positionen 14 und 18-20 für eine beliebige Aminosäure, an der Position 25 für T oder N und an der Position 27 für I oder F steht;

(b) L G X V G L I F X X S S L F S X V X X X X X L P V (SEQ ID NO 12)

wobei X an den Positionen 3 und 18-22 für eine hydrophobe Aminosäure (G, A, I, V, L, M, Y, F, W, P, S oder T), an der Position 10 für eine beliebige Aminosäure, an der Position 9 für L oder E und an der Position 16 für G oder N steht;

(c) L L L X I W G F X S Y X Y X (SEQ ID NO 13)

wobei X an den Positionen 9 und 12 für eine hydrophobe Aminosäure (G, A, I, V, L, M, Y, F, W, P, S oder T), an der Position 4 für S oder A und an der Position 14 für Q oder S steht.

Vorzugsweise umfaßt die Nukleinsäure die kodierende Sequenz einer der Sequenzen nach den SEQ ID NO 1, 2, 6, 7 oder 10, oder ein von diesen durch Substitution, Addition, Inversion und/oder Deletion einer oder mehrerer Basen abgeleitetes Derivat. In einer besonders bevorzugten Ausführungsform der Erfindung ist die Nukleinsäure eine DNA.

Gegenstand der Erfindung ist ebenfalls ein Fragment der erfindungsgemäßen Nukleinsäure, das in Antisinnorientierung zu einem Promotor die Expression eines Nukleobasentransporters in einer Wirtszelle hemmen kann. Dieses Fragment kann mindestens 10 Nukleotide, vorzugsweise mindestens 50 Nukleotide, ganz besonders bevorzugt mindestens 200 Nukleotide umfassen. Dieses Fragment kann in eine Wirtszelle eingebracht und dort in eine nicht-translatierbare RNA (Antisinn-RNA) transkribiert werden, die durch Bindung an ein endogenes Nukleobasentransporter-Gen oder an die daraus transkribierte mRNA deren Expression hemmen kann.

Ferner betrifft die Erfindung ein Konstrukt, das eine erfindungsgemäße Nukleinsäure und/oder ein erfindungsgemäßes Fragment derselben unter der Kontrolle von die Expression regulierenden Elementen enthält. Beispiele für solche regulierenden Elemente sind konstitutive oder induzierbare Promotoren, wie z.B. für Bakterien der *E. coli*-Promotor araBAD (Carra & Schlief, 1993, *EMBO J.* 12, 35-44), für Pilze der Hefepromotor PMA1 (Rentsch et al., 1995, *FEBS Lett.* 370, 264-268) und für Pflanzen der virale CaMV35S Promotor (Pietrzak et al., 1986, *Nucl. Acids Res.* 14, 5857-5868). Ferner kann die Nukleinsäure oder das Fragment mit einem Transkriptionsterminationssignal versehen werden. Derartige Elemente sind bereits beschrieben worden (siehe z.B. Gielen et al., 1984, *EMBO J.* 8, 23-29). Der transkriptionelle Startbereich kann sowohl nativ (homolog) als auch fremdartig (heterolog) zum Wirtsorganismus sein. Die Sequenz der Transkriptionsstart- und -terminationsregionen kann synthetisch hergestellt oder natürlich gewonnen sein oder eine Mischung aus synthetischen und natürlichen Bestandteilen enthalten. Vorzugsweise befindet sich die Nukleinsäure bzw. das Fragment in dem Konstrukt in Antisinnorientierung zum dem regulatorischen Element. Das Konstrukt kann

beispielsweise in das pflanzliche Genom eingeführt werden und nach seiner Transkription zur Unterdrückung der Bildung der pflanzeneigenen Nukleobasentransporter-Moleküle führen. In einer besonders bevorzugten Ausführungsform der Erfindung liegt das Konstrukt in einem Plasmid vor.

Das Plasmid kann ein Replikationssignal für *E. coli* oder Hefe und ein Marker-Gen enthalten, das die positive Selektion der mit dem Plasmid transformierten Wirtszellen erlaubt. Wird das Plasmid in eine pflanzliche Wirtszelle eingeführt, so können je nach Einführungsmethode weitere Sequenzen erforderlich sein, die dem Fachmann bekannt sind. Ist das erfindungsgemäße Plasmid beispielsweise ein Derivat des Ti- oder Ri-Plasmids, so muß die einzuführende Nukleinsäure bzw. das einzuführende Fragment von T-DNA-Sequenzen flankiert werden, die die Integration der Nukleinsäure bzw. des Fragments ins Pflanzengenom ermöglichen. Die Verwendung von T-DNA für die Transformation von Pflanzenzellen ist intensiv untersucht und u.a. in EP 120 516, Hoekema, The Binary Plant Vector System, Offset-drukkerij Kanters B.V. Ablasserdam, (1985), Kapitel V, Fraley et al., *Crit. Rev. Plant Sci.* 4, 1-46 und An et al., 1985, *EMBO J.* 4, 277-287 beschrieben worden. Ist die eingeführte Nukleinsäure bzw. das Fragment einmal im Genom integriert, so sind sie dort in der Regel stabil und bleiben auch in den Nachkommen der ursprünglich transformierten Zelle erhalten. Die integrierte Sequenz kann ebenfalls einen Selektionsmarker enthalten, der den transformierten Pflanzenzellen Resistenz gegenüber einem Biozid oder einem Antibiotikum, wie z.B. Kanamycin, G418, Bleomycin, Hygromycin oder Phosphinotricin vermittelt. Der individuell verwendete Marker sollte daher die Selektion transformierter Zellen gegenüber Zellen gestatten, denen die eingefügte DNA fehlt.

Gegenstand der Erfindung ist ferner eine Wirtszelle, die eine erfindungsgemäße Nukleinsäure und/oder eine Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder ein Fragment der vorgenannten Nukleinsäuren und/oder ein erfindungsgemäßes Konstrukt enthält. Die Wirtszelle gemäß der vorliegenden Erfindung kann aus Bakterien, Hefe-, Säuger- und Pflanzenzellen ausgewählt werden.

Ferner betrifft die vorliegende Erfindung eine transgene Pflanze sowie Pflanzenteile und/oder Samen dieser Pflanze, die eine erfindungsgemäße Nukleinsäure oder eine

Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder ein Fragment der vorgenannten Nukleinsäuren und/oder ein erfindungsgemäßes Konstrukt enthalten. Vorzugsweise ist die Nukleinsäure, das Fragment und/oder das Konstrukt an einer Stelle des Genoms integriert, die nicht seiner natürlichen Position entspricht.

Die vorliegende Erfindung betrifft ebenfalls ein Protein, das durch Expression einer erfindungsgemäßen Nukleinsäure oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 in einer Wirtszelle erhältlich ist. Die jeweiligen Initiationscodons (ATG) der Sequenzen Nrn. 1 bis 7 wurden im Sequenzprotokoll durch Unterstreichung gekennzeichnet. Vorzugsweise besitzt das Protein die gleichen Nukleobasentransporteigenschaften wie das von der SEQ ID NO 1 oder der SEQ ID NO 9 kodierte Protein. Zum Nachweis der Aktivität eines solchen Proteins können beispielsweise Aufnahmeexperimente durchgeführt werden, wie im Ausführungsbeispiel beschrieben. Antikörper, die mit einem erfindungsgemäßen Protein reagieren, werden von der Erfindung ebenfalls umfaßt.

Eine weitere Aufgabe der Erfindung besteht in der Bereitstellung eines Verfahrens zum Herstellen einer transgenen Pflanze. Diese Aufgabe wird durch ein Verfahren gelöst, das folgende Schritte umfaßt:

- Einbringen einer erfindungsgemäßen Nukleinsäure oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder eines erfindungsgemäßen Fragments der vorgenannten Nukleinsäuren in eine Pflanzenzelle; und

- Regenerieren einer Pflanze aus der transformierten Pflanzenzelle.

Transgene Pflanzen, die gemäß dem erfindungsgemäßen Verfahren hergestellt werden, können z.B. von Tabak-, Kartoffel-, Tomaten-, Zuckerrüben-, Sojabohnen-, Kaffee-, Erbsen-, Bohnen-, Baumwoll-, Reis- oder Maispflanzen abgeleitet sein.

Für das Einbringen der Nukleinsäure bzw. des Fragments in eine Pflanzenzelle stehen neben der Transformation mit Hilfe von Agrobakterien noch zahlreiche weitere Techniken zur Verfügung. Diese Techniken umfassen die Fusion von Protoplasten, die Mikroinjektion von DNA, die Elektroporation sowie ballistische Methoden und Virusinfektion.

Aus den transformierten Pflanzenzellen können dann in einem geeigneten Medium, welches Antibiotika oder Biozide zur Selektion enthalten kann, wieder ganze Pflanzen regeneriert werden. Die so erhaltenen Pflanzen können dann auf Anwesenheit der eingeführten DNA getestet werden.

Anders als bei der Transformation mit Hilfe von Agrobakterien, werden bei der Injektion und Elektroporation an sich keine speziellen Anforderungen an den Vektor gestellt. Es können einfache Plasmide wie z.B. pUC-Derivate verwendet werden. Sollen aber aus derartig transformierten Zellen ganze Pflanzen regeneriert werden, ist die Anwesenheit eines selektierbaren Markergens vorteilhaft. Die transformierten Zellen wachsen innerhalb der Pflanzen in der üblichen Weise (siehe auch McCormick et al., 1986, *Plant Cell Reports* 5, 81-84). Diese Pflanzen können wie üblich angezogen werden und mit Pflanzen, die die gleiche transformierte Erbanlage oder andere Erbanlagen besitzen, gekreuzt werden. Die daraus entstehenden hybriden Individuen haben die entsprechenden phänotypischen Eigenschaften.

Gegenstand der vorliegenden Erfindung ist ebenfalls ein Verfahren zum Beeinflussen der Nukleobasentransportereigenschaften einer Pflanze, eines Pflanzenteils, einer Pflanzenzelle und/oder von Samen, das den folgenden Schritt umfaßt:

- Einbringen einer erfindungsgemäßen Nukleinsäure oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder eines erfindungsgemäßen Fragments der vorgenannten Nukleinsäuren in eine Pflanzenzelle und/oder eine Pflanze.

Zur Beeinflussung der Nukleobasentransportereigenschaften einer Pflanze eignen sich sowohl Veränderungen der Spezifität des Transportsystems, die den Transport neuer Verbindungen ermöglichen, als auch solche die eine Änderung des Transportmechanismus hervorrufen. Beispielsweise sind Veränderungen möglich, die die Affinität oder Substratspezifität des Transporters dahingehend verändern, daß es zu einem effizienteren Nukleobasentransport in die Blätter oder zu einer Veränderungen der apikalen Dominanz, des Blühverhaltens oder der Seneszenz kommt, oder daß eine verbesserte Verteilung von Pestiziden ermöglicht wird.

Die erfindungsgemäßen Pflanzenzellen können zur Regeneration und Herstellung ganzer Pflanzen verwendet werden. Die Nukleinsäure gemäß der Erfindung sowie Nukleinsäuren mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 können dazu verwendet werden, homologe Sequenzen aus Bakterien, Pilzen, Pflanzen, Tieren und/oder Menschen zu isolieren. Um nach homologen Sequenzen suchen zu können, müssen zunächst Genbanken angelegt werden, die für den Gehalt an Genen eines Organismus oder für die Expression von Genen in diesem Organismus repräsentativ sind. Erstere sind genomische, letztere sind cDNA-Banken. Aus diesen können mit Hilfe einer Sonde aus den vorgenannten Nukleinsäuren verwandte Sequenzen isoliert werden. Hat man die dazugehörigen Gene identifiziert und isoliert, ist eine Bestimmung der Sequenz und eine Analyse der Eigenschaften der von dieser Sequenz kodierten Proteine möglich.

Eine weitere Verwendung der vorgenannten Nukleinsäuren betrifft die Expression eines Nukleobasentransporters in pro- und/oder eukaryontischen Zellen. Werden die vorgenannten Nukleinsäuren in eine prokaryontische Zelle eingeführt, so wird eine von Bakterien translatierbare RNA-Sequenz eines eukaryontischen Nukleobasentransporters gebildet, die trotz der erheblichen Unterschiede in den Membranstrukturen von Pro- und Eukaryonten in einen funktionalen eukaryontischen Nukleobasentransporter mit dessen Substratspezifizität translatiert wird. Dies ermöglicht die Verwendung von Bakterienstämmen für Studien der Eigenschaften des Transporters sowie seiner Substrate. Die vorgenannten Nukleinsäuren können ebenfalls unter der Kontrolle eines regulatorischen Elements in Antisinnorientierung zur Hemmung der Expression eines endogenen Nukleobasentransporters in pro- und/oder eukaryontischen Zellen verwendet werden. Die Herstellung transgener Nutzpflanzen stellt eine weitere Verwendungsmöglichkeit dieser Nukleinsäuren dar.

Weitere Verwendungen sind:

- Wenn die Transporter essentiell für die Funktion der Pflanze sind, können sie als Herbizidtarget dienen: Screeningverfahren in Hefe um nach Inhibitoren zu suchen, diese können im Hefesystem und durch chemische Modifikation optimiert werden und dann an Pflanzen getestet werden.

- Da Substitutionen am Substratgrundgerüst erlaubt sind, kann man die Transporter nutzen, um Pestizide zu mobilisieren, z.B. durch Anhängen von Purin oder Pyrimidinresten an Fungizid oder Insektizid.

Spezielle Verwendungen sind:

1. Substratklasse A: Transporter sind verantwortlich für den Transport von Sekundärmetaboliten bzw. Alkaloiden wie z.B. Koffein, Theobromin, Nikotin und ähnliche Substanzen.

- Überexpression oder ektopische Expression unter Kontrolle verschiedener Promotoren z.B. CaMV-35S oder spezifische Promotoren um Sekundärmetabolite dieser Stoffklasse besser in bestimmte Organe, z.B. Blätter zur Ernte, Samen und Knollen oder Rüben zu transportieren.

- Cosuppression oder Antisense-Repression um den Gehalt an derartigen Sekundärmetaboliten, speziell toxischer Substanzen in bestimmten Organen, z.B. Nahrungsprodukten zu verringern; z.B. als neuartiges Decaffeinierungs-Verfahren.

- Sekundärmetabolite sind wichtige Abwehrstoffe der Pflanze gegen Infektionen und Tierfraß. Eine verbesserte Versorgung von betroffenen Organen kann zu verbesserter Resistenz und Widerstandskraft führen.

- Sekundärmetabolite als pharmazeutische Produkte (Pflanze als Bioreaktor). Eine optimale Versorgung von Ernteorganen ist essentiell für die Extrahierbarkeit und kann durch optimierten Transport in transgenen Pflanzen erreicht werden.

2. Substratklasse B: Transporter sind verantwortlich für den Transport von Nukleobasen und Derivaten, z.B. einerseits Adenin, Xanthin, Allantoin, Allantoat, Hypoxanthin, Urat, Xanthosin, Inosin; andererseits Cytosin und seine Derivate wie Barbiturate und Folsäure, aber auch Nukleoside wie Adenosin etc. Umsteuerung von Transportprozessen in transgenen Pflanzen führen zu

- veränderter Zellteilungsaktivität und verbesserter Entwicklung autonomer Zellen: Nukleobasen spielen bei der DNA- und RNA-Synthese und damit der Zellteilungsaktivität eine wichtige Rolle, beispielsweise wird Pollen mit Nukleobasen versorgt. Einsatz zur Erzeugung männlich steriler Pollen durch Transportinhibition;

- bei dem Transport von Allantoinsäure eine Rolle bei der Fixierung von atmosphärischem Stickstoff. Interzellulärer Transport ist wichtig für die Stickstoffassimilation der Pflanze.

- Adenin ist ein essentieller Baustein von ATP. Die externe Versorgung z.B. des Phloems mit ATP könnte durch Veränderung der Expression von PuP Transportern positiv oder negativ in transgenen Pflanzen beeinflußt werden.

3. Substratklasse C: Transporter sind verantwortlich für den Transport von Pflanzenhormonen, z.B. Cytokininen und Cytokininderivaten, z.B. Ribosiden (siehe Adenin/Adenosin).

Da Cytokinine eine zentrale Rolle in der Steuerung von Entwicklungs- und Stoffwechselprozessen spielen, kann eine Änderung der Aktivität durch

- Überexpression, ektopische Expression oder Repression (durch Cosuppression oder Antisense) in transgenen Pflanzen zu verbesserten Erträgen (Steuerung von sink-source-Relationen), verändertem Verzweigungsgrad (Rolle in der apikalen Dominanz), Verbesserung des Keimungsverhaltens von Samen, Beschleunigung oder Verlangsamung der Knospenbildung und Beschleunigung oder Verlangsamung der Seneszenz (Cytokinine verlangsamen die Seneszenz) führen. Außerdem wird die Zellteilungsaktivität und damit die Bildung von Seitenwurzeln, die Größe und Kapazität von Ertragsorganen, die Morphogenese in Gewebekultur, die Expansion von Blättern und die Regulation der Wassereffizienz aufgrund des Einflusses auf Stomaöffnung und Plastidenentwicklung beeinflußt.

4. Transport von verwandten Substanzen: Auch Auxine stehen strukturell dem Spektrum der von NukleobasenTransportern vermittelten Substraten nahe und könnten

ebenfalls durch die Transporter vermittelt werden. Bei einer Veränderung des Transports von Auxinen kann das gesamte Spektrum der Auxinwirkungen beeinflusst werden.

5. Modifikation der Transporteigenschaften der Transporter durch Mutagenese: Erweiterung des Substratspektrums, z.B. in Richtung des Transports von Alkaloiden, die bisher von den Transportern nicht erkannt werden, synthetischen Hormonen, die schlecht transportiert werden, Nukleobasen die nicht effizient genug transportiert werden als Ausgangsbasis zur Veränderung von Transportprozessen in transgenen Pflanzen.

Bisher konnten zu fast allen neuen pflanzlichen Transportern in tierischen/menschlichen Genomen Homologe gefunden werden (Beispiele: Glukosetransporter der pflanzlichen MST-Familie sind den tierischen GLUT-Transportern auf Sequenzebene verwandt; Aminosäuretransporter der AAP-Familie wurden später in Tieren gefunden (VGAT, SN1); Aminosäuretransporter der CAT Familie in Tieren und Pflanzen sind verwandt). Auf Basis der Ähnlichkeit der biochemischen Eigenschaften, die für den tierischen/menschlichen Transport von Adenin und die Kompetition durch Koffein gefunden wurde, wird postuliert, daß ein bisher noch nicht identifiziertes Homolog der PUP-Familie in diesen Systemen für den Nukleobasen/Nukleosid und Derivattransport verantwortlich ist. Die Expression dieses Systems in Hefezellen zum Funktionsnachweis ermöglicht erstens die Beschreibung, zweitens die Identifizierung von möglichen Erbkrankheiten, die durch Defekte in dem System hervorgerufen werden, die Identifizierung von neuen Leitstrukturen für Pharmazeutika und die Beeinflußung des Transports von Substanzen über die Blut/Hirnschranke, da der Adenin/Koffeintransport an dieser Stelle vorkommt.

Die folgenden Figuren und Beispiele erläutern die Erfindung.

Figur 1 zeigt eine Hydrophobizitätsanalyse des PUP-Nukleobasentransporterproteins nach Kyte und Doolittle.

Figur 2 zeigt die in Form eines Diagramms dargestellten Ergebnisse eines Aufnahmeexperiments, bei dem die Cytosinaufnahmerate des Hefestamms

MG877ura3⁻::pFL61-PUP1 und des Wildtypstamms Σ1278b (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) bei verschiedenen pH-Werten gemessen wurde. Die Substratkonzentration betrug 100 µM.

Figur 3 zeigt die in Form eines Diagramms dargestellten Ergebnisse eines Aufnahmeexperiments, bei dem die Cytosinaufnahme des Hefestamms MG877ura3⁻::pFL61-PUP1 mit und ohne Zugabe von Glukose gemessen wurde. Die Zellen wurden vor Beginn der Aufnahmemessung zweimal mit Wasser gewaschen und 30 Minuten lang bei Raumtemperatur inkubiert. Fünf Minuten nach Beginn der Messungen wurde einem der Versuchsansätze Glukose bis zu einer Endkonzentration von 1% zugesetzt.

Figur 4 zeigt eine Analyse der Substratspezifität des in Hefe exprimierten PUP1-Nukleobasentransporters (schwarze Balken) im Vergleich zu dem Hefe-eigenen FCY2-Transporter (weiße Balken).

Figur 5 zeigt die in Form eines Diagramms dargestellten Ergebnisse eines Aufnahmeexperiments, bei dem die Cytokininaufnahme des Hefestamms MG877ura3⁻::PUP1 mittels ^{3}H-markiertem Zeatin untersucht wurde. Angegeben ist die Aufnahme pro Zeit bei einer Konzentration von 100 µM trans-Zeatin. In diesem Fall wurde PUP1 unter Kontrolle des Hefe-ATPase Promotors PMA1 im Vektor pDR195 (Rentsch et al.,1995, *FEBS Lett.* 370, 264-368) in der Mutante MG877ura3⁻ (FCY2) exprimiert. Als Kontrolle diente der leere Vektor (FCY2pDR195).

Figur 6a zeigt eine schematische Darstellung des Plasmids p35S-PUP1, ein Derivat des Plasmids pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8721). A steht für ein Fragment aus dem Genom des Blumenkohlmosaik-Virus, das den 35S-Promotor (nt 6909-7437) trägt. Das Promotor-Fragment wurde als *EcoRI/KpnI*-Fragment aus dem Plasmid pDH51 (Pietrzak et al., *Nucl. Acids Res.* 14, 5857-5868) präpariert. B steht für ein *NotI/NotI*-Fragment der cDNA, flankiert von einer Polylinkerregion aus pT7T3 18U/*NotI* mit *KpnI* und *XbaI*-Schnittstellen mit der kodierenden Region des Nukleobasentransporters von *Arabidopsis thaliana* in Sinnorientierung zum Fragment A. Der in Fragment B eingezeichnete Pfeil gibt die Leserichtung der cDNA an. C steht für ein Polyadenylierungssignal des Gens 3 der T-DNA des Plasmids pTiACH5 (Gielen et

al., 1984 *EMBO J.* 3, 835-846), Nukleotide 11749 bis 11939, welches als *PvuII/HindIII*-Fragment aus dem Plasmid pAGV40 (Herrera-Estrella et al., 1983, *Nature* 303, 209-213) isoliert und nach Addition eines *SphI*-Linkers an die *PvuII*-Schnittstelle zwischen die *SphI*- und die *HindIII*-Schnittstelle des Polylinkers von pBIN19 kloniert wurde.

Figur 6b zeigt eine schematische Darstellung des Plasmids p35S-α-PUP1, ein Derivat des Plasmids pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8721). A und C stehen jeweils für den CaMV35S-Promotor und das Polyadenylierungssignal des Gens 3 der T-DNA des Plasmids pTiACH5 (siehe Figur 6a). B steht für ein *SspI/HpaI*-Fragment der cDNA mit der kodierenden Region des Nukleobasentransporters von *Arabidopsis thaliana* in Antisinnorientierung zum Fragment A. Der in Fragment B eingezeichnete Pfeil gibt die Leserichtung der cDNA an.

Figur 7 zeigt die Messung von einwärts gerichteten Strömen in *Xenopus*-Oozyten, die PUP1 exprimieren, bei einer Haltespannung von - 80mV. Es werden einwärts gerichtete Ströme gemessen, sobald Adenin zugegeben wird. Die Stromstärke ist konzentrationsabhängig (Balken geben auf der Ordinate den Strom und auf der Abszisse die Zeit an).

Figur 8 zeigt: (A) das Wachstum von DM734-284DpDR195 auf 150 µM Adenosin (Kontrolle) und (B) das Wachstum von DM734-284DpDR195PuP1 auf 150 µM Adenosin.

Figur 9 zeigt das Wachstum von *Saccharomyces cerevisiae* MG877ura3$^-$ (K) und mit dem Konstrukt pDR195PUP1 (1) bzw. mit dem Konstrukt pDR195PUP2 transformierten Klonen auf Koffein-Platten mit verschiedenen Koffein-Konzentrationen.

BEISPIELE

Allgemeine Methoden

a) Klonierungsverfahren: Zur Klonierung in *E. coli* wurde der Vektor pT7T3 18U (Pharmacia) und zur Transformation von Hefen der Vektor pFL61 (Minet & Lacroute, 1990, *Curr. Genet.*18, 287-291) eingesetzt. Zur Pflanzentransformation wurden die Gen-

konstruktionen in den binären Vektor pBinAR, ein Derivat von pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8721), kloniert.

b) Bakterien- und Hefestämme: Für die pT7T3 18U- und pFL61-Vektoren sowie für die pBinAR-Konstrukte wurde der *E. coli*-Stamm DH5α verwendet. Als Ausgangsstamm für die Expression der cDNA-Bibliothek in Hefe wurde der Hefestamm MG887 (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) mit der Mutation *fcy2* verwendet, nachdem eine ura3-Defizienz eingeführt worden war.

c) Transformation von *Agrobacterium tumefaciens*: Der DNA-Transfer in die Agrobakterien erfolgte durch direkte Transformation nach der Methode von Höfgen und Willmitzer (1988, *Nucl. Acids Res.* 16, 9877). Die Plasmid-DNA transformierter Agrobakterien wurden nach der Methode von Birnboim und Doly (1979, *Nucl. Acids Res.* 7, 1513-1523) isoliert und nach geeigneter Restriktionsspaltung gelelektrophoretisch analysiert.

d) Pflanzentransformation: Die Pflanzentransformationen kann durch *Agrobacterium tumefaciens* (Stamm C58C1, pGV2260)-vermittelten Gentransfer erfolgen (Deblaere et al., 1985, *Nucl. Acids Res.* 13, 4777-4788). Die Transformation von *A. thaliana* wird beispielsweise mittels Vakuum-Infiltration (modifiziert nach Bechtold et al. (1993) Comptes Rendus de l'Academie des Sciences Serie III, Sciences de la Vie 316: 1194-1199) ausgeführt. Töpfe (Durchmesser 10 cm) werden mit Erde gefüllt und anschließend mit einem Fliegennetz überspannt. Auf diesem Netz werden *A. thaliana*-Samen ausgesät. Sechs bis acht Wochen nach dem Aussäen wurden die Pflanzen für die Vakuum-Infiltration benutzt. Zur Vakuum-Infiltration wurden von den entsprechenden *Agrobacterium*-Stämmen 2 x 1 Liter Kulturen in YEB + Antibiotikum (50 µg/ml Kan und 100 µg/ml Rif) bei 28°C angezogen. Bei einer OD_{600} von 1,5 werden die Zellen bei 3000 g geerntet und in 600 ml Infiltrationsmedium (0,5 x MS Medium (Sigma), 5% Saccharose, 44 µM Benzylaminopurin) resuspendiert. Die Bakteriensuspension wird in 250 ml Weckgläser gefüllt und in einen Exsikkator gestellt. Die *A. thaliana*-Pflanzen werden "kopfüber" in die Bakteriensuspension getaucht, dann wird für 5 min Vakuum angelegt. Nach 3-4 Wochen werden die Samen dieser Pflanzen geerntet. Zur Oberflächensterilisation werden die Samen 10 min lang in 4% Natriumhypochlorid, 0,02% Triton geschüttelt, bei 1500 g abzentrifugiert, viermal mit sterilem Wasser gewaschen

und in 3 ml 0.05% Agarose pro 5000 Samen resuspendiert. Die Samen-Agarose-Lösung wird auf MSS Medium (1 x MS, 1% Saccharose, 0,8% Agarose, 50 µg/ml Kanamycin, pH 5,8) ausgebreitet (Platten mit 13,5 cm Durchmesser für 5000 Samen). Zur Reduzierung des Feuchtigkeitsverlustes werden die Platten mit Parafilm® verschlossen. Die Kanamycin-resistenten Pflanzen werden in Erde umgesetzt. Samen dieser Pflanzen werden geerntet und analysiert.

e) Nachweis der Nukleobasentransporter-Aktivität: Der Aktivitätsnachweis kann beispielsweise durch Aufnahmeexperimente mit radioaktiven Substraten (z.B. [^{14}C]-Adenin) in die mit dem pflanzlichen Transportergen unter Kontrolle des Hefepromotors transformierten *fcy2*-Hefemutante (MG877ura3$^-$::pFL61-PUP1), prinzipiell wie bei Ninnemann et al., 1994 (*EMBO J.* 15, 3464-3471) beschrieben, durchgeführt werden. Alternativ können andere Expressionsysteme, z.B. *Xenopus*-Oocyten (Boorer et al., 1996, *J. Biol. Chem.* 271, 2213-2220), unter Verwendung elektrophysiologischer Meßmethoden eingesetzt werden.

Beispiel 1: Klonierung des PUP1-Nukleobasentransportergens aus *Arabidopsis thaliana*

Die Klonierung des PUP1-Nukleobasentransporters erfolgt durch Komplementation des Hefe-Stamms MG887ura3$^-$ (*fcy2*) (diese Arbeit; Vorstufe MG887 Grenson 1969, *Eur. J. Biochem.* 11, 249-260) mit einer cDNA-Genbank aus *Arabidopsis thaliana* und Selektion der Nukleobasentransporter-positiven Zellen. Die *fcy2*-Hefemutante kann in Medien mit Adenin oder Cytosin als einziger Stickstoffquelle nicht wachsen (Grenson, 1969, *Eur. J. Biochem.* 11, 249-260; Polak & Grenson, 1973, *Eur. J. Biochem.* 32, 276-282). Zur Einführung eines Auxotrophiemarkers (ura3$^-$) wurde die Nukleobasenaufnahme-defiziente Mutante MG887 (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) mit einem Fragment des URA3-Gens transformiert, welches eine interne Deletion trägt. Durch Selektion auf der toxischen Vorstufe 5-Fluoroorotat konnte eine URA3-defiziente Mutante MG887ura3$^-$ isoliert werden.

Zur Komplementation der Nukleobasentransport-Mutation des Hefestamms MG887ura3$^-$ wird im Hefe-Expressionsvektor pFL61 (Minet & Lacroute, 1990, *Curr. Genet.* 18, 287-291) klonierte cDNA aus jungen Keimlingen von *Arabidopsis thaliana* (Zwei-

Blatt-Stadium) verwendet (Minet et al., 1992, *Plant J.* 2, 417-722). Etwa 1 µg des Vektors mit einer cDNA-Insertion wurden in den Hefestamm MG887ura3$^-$ nach der Methode von Dohmen et al. (1991, *Yeast* 7, 691-692) transformiert. Hefetransformanten, die auf Minimalmedium mit 1 mM Adenin als einziger Stickstoffquelle wachsen konnten, wurden vermehrt. Aus diesen Klonen wurde Plasmid-DNA nach Standardverfahren isoliert. Der Stamm MG887ura3$^-$ wurde erneut mit dem isolierten Plasmid transformiert. Auf diese Weise wurde ein Plasmid pFL61-PUP1 erhalten, das die *fcy2*-Mutation komplementieren kann. Dieses Plasmid hat eine Insertion mit einer Größe von 1,2 kb, die das PUP1- Nukleobasentransportergen enthält.

Der durch Transformation von MG887ura3$^-$ mit dem Plasmid pFL61-PUP1 erhaltene Hefestamm MG887ura3$^-$::pFL61-PUP1 wird für Aufnahmestudien mit Adenin oder Nukleobasen verwendet. Durch gentechnische Veränderung der Kodierregion des Nukleobasentransportergens PUP1 nach Standardverfahren (vgl. Sambrook et al., 1989, Molecular cloning: A laboratory manual, 2. Aufl., Cold Spring Harbor Laboratory Press, NY, USA) können dessen Spezifität bzw. die Eigenschaften des Transportmechanismus verändert werden. Der Stamm MG887ura3$^-$::pFL61-PUP1 eignet sich direkt für die Untersuchung von Inhibitoren oder Promotoren des Nukleobasentransports.

Das von dem Hefestamm M887ura3$^-$::pFL61-PUP1 exprimierte PUP1-Protein wurde einer Hydrophobizitätsanalyse nach Kyte und Doolittle unterzogen. Wie aus Fig. 1 ersichtlich, weist das PUP1-Protein 9-12 stark hydrophobe Regionen (positive Werte auf der Y-Achse) auf, die lang genug sind, um jeweils einmal die Membran zu durchspannen. Die erste hydrophobe Region ist nicht bei allen PUP-Proteinen konserviert.

Sequenzanalyse der cDNA-Insertion des Plasmids pFL61-PUP1

Aus dem Hefe-Stamms MG887ura3$^-$::pFL61-PUP1 wurde das Plasmid pFL61-PUP1 isoliert und mit Hilfe synthetischer Oligonukleotide wurde die Insertion nach der Methode von Sanger et al. (1977, *Proc. Natl. Acad. Sci. USA* 74, 5463-5467) sequenziert. Die kodierende Sequenz des PUP1-Gens wird in SEQ ID NO 1 und die davon abgeleitete Proteinsequenz in SEQ ID NO 8 wiedergegeben.

Beispiel 2: Aufnahmestudien mit ^{14}C-markiertem Adenin, ^{14}C-markiertem Cytosin und ^{3}H-markierten Cytokininen an dem Hefe-Stamm MG887ura3^{-}::pFL61-PUP1

Für die Messung der Aufnahmeraten wurden die Hefe-Stämme MG887ura3^{-}::pFL61, MG887ura3^{-}::pFL61-PUP1 und deren Ausgangsstamm Σ1278b (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) in Vollmedium (YPD) ohne Uracil und mit 2% Glukose als Kohlenstoffquelle bis zu einer OD_{600} von 0,6 bei 28°C angezogen. Die Zellen wurden bei 3000 g geerntet, zweimal mit Wasser gewaschen und mit Natriumphosphatpuffer (100 mM, pH 4,5), 1% Glukose auf eine OD600 von 12 eingestellt. Die Zellsuspension wurde in 100 µl Portionen bis zum Beginn des Aufnahmeexperiments auf Eis gelagert. Vor dem Start der Aufnahmemessungen wurden die Zellen für 2 min bei 30°C vorinkubiert. Die Reaktion wurde dann gestartet, indem zu 100 µl Zellsuspension 100 µl der radioaktiv markierten Substratlösung gegeben wurde. Das Reaktionsgemisch wurde bei 30°C inkubiert und nach 30, 60, 120, 180 Sekunden wurde jeweils 50 µl der Suspension entnommen und in 4 ml eiskaltes Wasser gegeben (bei Messungen über 180 Sekunden wurde dementsprechend mehr Zellsuspension und Substratlösung eingesetzt). Die Zellen wurden auf Glasfiberfilter gesaugt und mit 4 ml eiskaltem Wasser gewaschen. Die Radioaktivität auf den Filtern wurde anschließend im Flüssigkeits-Szintillationszähler ermittelt.

Substratlösung: Bei der Substratlösung handelt es sich um einen 100 mM Natriumphosphatpuffer pH 4,5 mit 1% Glukose und 9,25 Bq/µl des radioaktiv markierten Substrats (25-100 µM [^{14}C]-Adenin oder [^{14}C]-Cytosin). Darüber hinaus enthält die Substratlösung das nicht markierte Substrat, eventuelle Inhibitoren und Kompetitoren in 2facher Endkonzentration. Zur Bestimmung des pH-Optimums wurde auch der pH-Wert des Natriumphosphatpuffers verändert. Bei der Messung des Einflusses von Glukose auf die Aufnahmeraten enthielt weder die Zellsuspension noch die Substratlösung Glukose. Glukose wurde erst nach dem Start der Aufnahmemessungen zum Meßansatz in einer Endkonzentration von 1% hinzugefügt. Weiterhin konnte gezeigt werden, daß auch PUP2 in der Lage ist, Adenin zu transportieren.

In Tabelle 1 wird die durch den PUP1-Nukleobasentransporter vermittelte Aufnahme von radioaktiv markiertem Adenin und Cytosin durch den Hefestamm MG887ura3^{-}::pFL61-

PUP1 angegeben. Zur Berechnung der intrazellulären Konzentration wurde das Zellvolumen auf das vierfache des Trockengewichts geschätzt (Ninnemann et al, 1994, *EMBO J.*15, 3464-3471). Die Aufnahme erfolgt entgegen einem Konzentrationsgradienten.

Tabelle 1

	Anfangskonz. Medium	*Endkonz. Medium*	*Endkonz. Zellen*	*Anreicherungs-faktor*
Adenin	200 µM	155 µM	2350 µM	15
Cytosin	200 µM	145 µM	2660 µM	18

Der Einfluß von verschiedenen Inhibitoren auf die durch den PUP1-Nukleobasentransporter vermittelte Adenin- bzw. Cytosinaufnahme in dem Hefestamm MG887ura3$^-$::pFL61-PUP1 wird in Tabelle 2 gezeigt. Die Inhibitoren wurden fünf Minuten vor Anfang der Messungen zu den Zellen hinzugegeben. Die Protonophoren Carbonyl-Cyanid-m-Chlorphenylhydratzon (CCCP) und 2,4-Dinitrophenol (2,4-DNP) und der H^+/ATPase-Inhibitor Diethylstilbestrol blockieren die Aufnahme. Dies kann als klarer Hinweis auf einen sekundäraktiven, protonengekoppelten Aufnahmemechanismus gewertet werden.

Tabelle 2

Inhibitor	*relative Adeninaufnahme [%]*	*relative Cytosinaufnahme [%]*
ohne Inhibitor	100	100
100µM Diethylstilbestrol	4	8
100µM DCCD	47	55
100µM CCCP	13	20
100µM 2,4-DNP	54	58
10µg/ml Cycloheximid	95	97

Figur 2 zeigt, daß die durch den PUP1 Nukleobasentransporter vermittelte Cytosinaufnahme in den Hefestamm MG877ura3$^-$::pFL61-PUP1 von dem pH-Wert abhängig ist. Ähnliche Ergebnisse wurden für die Adeninaufnahme erzielt (Daten nicht gezeigt).

Wie in Fig. 3 gezeigt, ist die durch den PUP1-Nukleobasentransporter vermittelte Cytosinaufnahme in dem Hefestamm MG877ura3$^-$::pFL61-PUP1 glukoseabhängig. Ähnliche Ergebnisse wurden für die Adeninaufnahme erzielt (Daten nicht gezeigt). Die Aktivitätszunahme bei Glukosezugabe kann als Hinweis auf eine Energieabhängigkeit gewertet werden. Dieses so wie die beobachtete Aktivitätszunahme bei abnehmendem pH-Wert deutet auf einen sekundäraktiven, protonengekoppelten Aufnahmemechanismus hin.

Zur Untersuchung der Subtratspezifität des in Hefe exprimierten PUP1-Nukleobasentransporters im Vergleich zu dem Hefe-eigenen FCY2-Transporter wurden Kompetitionsexperimente mit nicht-radioaktiven Subtraten durchgeführt. Die Ergebnisse dieser Experimente werden in Fig. 4 gezeigt. Die Aufnahme von radioaktiv markiertem Adenin wurde gemessen und als 100% gesetzt (entspricht 1,7 bzw. 0,9 nmol.min^{-1}.mg^{-1} Trockengewicht). Die Messungen wurden bei einer Substratkonzentration von 25 µM durchgeführt. Die Kompetitoren wurden in 10fachem molarem Überschuß eingesetzt.

Zur Analyse der kompetitiven Inhibition des durch den PUP1-Nukleobasentransporters vermittelten Adenintransports durch die Cytokinine Kinetin und Zeatin wurden Aufnahmeversuche mit verschiedenen Konzentrationen von radioaktiv markiertem Adenin sowie unterschiedlichen Kompetitor-Konzentrationen durchgeführt. Aus Lineweaver/ Burk-Auftragungen wurden die Inhibitor-Konstanten Ki ermittelt (35 µM für Kinetin und 30 µM für Zeatin).

Direkte Aufnahmeexperimente in PUP1-exprimierenden Hefezellen mit ^{3}H-markierten Cytokininen (Zeatin und 2-Isopentenyladenin) zeigen, daß PUP1 auch Cytokinine transportieren kann (Figur 5). Die Aufnahmeexperimente wurde mit ^{3}H-markiertem Zeatin in MG887ura3$^-$ wie für Adenin durchgeführt. Pro Reaktionsansatz wurden 87,3 Bq/µl des radioaktiven Substrates bei einer Gesamtkonzentration von 100 µM trans-Zeatin eingesetzt. Die Ergebnisse von drei unabhängigen Experimenten zeigten für die PUP1-exprimierenden Hefe-Klone gegenüber der mit dem Kontrollplasmid pDR195 transformierten Kontrollstämme eine 70-fach erhöhte Aufnahme an radioaktiv markiertem trans-Zeatin. Dies zeigt, daß PUP1 den Transport von trans-Zeatin vermittelt.

Beispiel 3: Transformation von Pflanzen mit Konstruktionen zur Überexpression bzw. Antisinn-Repression eines Nukleobasentransportergens

Das Einbringen einer für einen pflanzlichen Nukleobasentransporter kodierenden Nukleinsäure und die Überexpression bzw. die Antisinn-Repression eines Nukleobasentransportergens zur Veränderung des Transports von Nukleobasen und ihren Derivaten wird hier am Beispiel von *Arabidopsis thaliana* beschrieben. Die Anwendung ist aber nicht auf diese Spezies beschränkt.

a) Überexpression: Das 1.2kb *NotI*-Fragment aus dem Plasmid pFL61-PUP1, das als Insertion die cDNA für einen Nukleobasentransporter von *Arabidopsis thaliana* enthält, wurde in den mit *NotI* geschnittenen Vektor pT7T3 18U/*NotI* kloniert. Der Vektor pT7T3 18U/*NotI* wurde erzeugt, indem in die *SmaI*-Schnittstelle von pT7T3 18U (Pharmacia) ein *NotI*-Linker eingefügt wurde. Die Orientierung des Fragments wurde durch eine Restriktionsspaltung überprüft. Der Vektor enthält eine *KpnI*- und eine *XbaI*-Schnittstelle in der multiplen Klonierungsstelle, so daß die PUP1-cDNA aus diesem Vektor als *KpnI-XbaI*-Fragment isoliert werden konnte. Dieses 1.2kb *KpnI-XbaI*-Fragment wurde in den *KpnI-XbaI* geschnittenen Vektor pBinAR, ein Derivat von pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8720), kloniert. Das resultierende Plasmid wurde p35S-PUP1 genannt.

b) Antisinn-Repression: Aus dem Plasmid pFL61-PUP1 wurde ein 1,1 kb großes *HpaI-SspI*-PUP1-Fragment isoliert und in Antisinn-Orientierung in die *SmaI*-Schnittstelle von pBinAR kloniert. Die Orientierung des Fragments wurde durch eine Restriktionsspaltung überprüft. Das resultierende Plasmid wurde p35-α-PUP1 genannt.

Die PUP1-cDNA Fragmente tragen in den Figuren 6a und 6b die Bezeichnung "B". Je nachdem, ob B in Sinnorientierung zum CaMV-35S-Promotor von pBinAR eingeführt wurde oder nicht, trägt das entstandene Plasmid die Bezeichnung p35S-PUP1 bzw. p35S-α-PUP1. Zwischen dessen *EcoRI*- und *KpnI*-Schnittstellen wurde ein Fragment aus dem Genom des Blumenkohlmosaik-Virus, das den 35S-Promotor (nt 6909-7437) trägt, eingefügt. Das Promotorfragment wurde als *EcoRI/KpnI*-Fragment aus dem Plasmid pDH51 (Pietrzak et al., *Nucl. Acids Res.* 14, 5857-5868) präpariert. In der Plasmidkarte trägt das Promotorfragment die Bezeichnung "A". Zwischen der *SphI*- und der

HindIII-Schnittstelle von pBinAR ist außerdem das Polyadenylierungssignal des Gens 3 der T-DNA des Plasmids pTiACH5 (Gielen et al., *EMBO J.* 3, 835-846) eingefügt. Hierzu war ein *PvuII/HindIII*-Fragment (nt 11749-11939) aus dem Plasmid pAGV 40 (Herrera-Estrella et al., 1983, *Nature* 303, 209-2139) an der *PvuII*-Schnittstelle mit einem *SphI*-Linker versehen worden. Das Polyadenylierungssignal trägt in der Plasmidkarte die Bezeichnung "C".

Nach Transformation von Agrobakterien mit den Plasmiden p35S-PUP1 und p35S-α-PUP1 wurden diese zur Vakuuminfiltration von *Arabidopsis thaliana* eingesetzt.

Zehn unabhängig erhaltene Transformanten für beide Konstrukte, in denen die Präsenz des intakten, nicht rearrangierten chimären Gens mit Hilfe von "Southern blot"- Analysen nachgewiesen worden war, wurden bezüglich der Veränderungen im Nukleobasen-transport untersucht.

Beispiel 4: Expression des PUP1-Gens in *Xenopus*-Oozyten

Die cDNA für PUP1 wurde mit NotI geschnitten und in einen Oozytenexpressionsvektor kloniert, der untranslatierte 5'- und 3'-Bereiche des ß-Globins aus *Xenopus* enthält. Die cDNA wurde dann mittels PCR vervielfältigt. Als Vorwärtsprimer wurde SP6 (aus dem Oozytenvektor) benutzt, als Rückwärtsprimer wurde ein Primer, der das STOP-Codon von PUP1 zerstört und gleichzeitig eine *NotI*-Schnittstelle einfügt, benutzt. Dieses PCR-Fragment wurde *NotI* und *HindIII* geschnitten und in einen Oozytenexpressionsvektor ligiert, der zusätzlich hinter der NotI-Schnittstelle das Gen für GFP („green floureszent protein" aus der Qualle) enthält. So entsteht ein offener Leserahmen für ein Fusions-protein aus PUP1 und GFP. Durch Einfügen der *NotI*-Schnittstelle entsteht ein Linker mit drei für Alanin codierende Basenpaare. Das Plasmid wurde mit *MluI* linearisiert, und RNA wurde *in vitro* mittels SP6-Polymerase transkribiert und zu etwa 1 ng/nl in Wasser aufgenommen. Je Oozyte (Reifestadium 5 bis 6) wurden 50 nl injiziert. Nach zweitägiger Inkubation bei 16° wurden Oozyten zum Messen in eine entsprechende Meßkammer gelegt und mit verschiedenen Lösungen überspült. Die Lösungen enthielten: 100 mM N-Methyl-D-Glutaminchlorid, 2 mM Calziumchlorid, 5 mM MES, pH 5,0 oder 7,3 und entsprechende Konzentrationen Adenin.

Zur Spannungsmessung wurde eine Glaselektrode (gefüllt mit 3 M KCl) in die Oozyte eingestochen. Mit einem Dagan-Verstärker wurde das Potential relativ zur Referenzelektrode gemessen. Diese war über eine Agarbrücke mit der Badlösung verbunden. In Lösungen ohne Adenin bei pH 7,3 hatten Oozyten ein typisches Ruhepotential von etwa – 30 mV (siehe Figur 7). Adenin in der Badlösung ruft eine Depolarisation der Oozyte hervor. Dies zeigt, daß PUP1 auch in Oocyten Adenin transportiert.

Zur Strommessung wurde die Oozyte auf eine Spannung von – 80 mV geklemmt. Adenin in der Badlösung ruft einen einwärtsgerichteten Strom hervor. Da Adenin selbst nicht geladen ist, deutet das auf einen Cotransport mit geladenen Ionen, z.B. Protonen, hin.

Beispiel 5: PUP1-vermittelter Transport von Adenosin in Hefe

Der *Saccharomyces cerevisiae*-Stamm DM734-284D (Genotype: *ade8-18, ade2-1, arg4-16, leu2-27, trp1-1, lys2, ura3*; Yeast Genetic Stock Center) wurde für Wachstumsstudien benutzt. Dieser Hefe-Stamm ist durch die Mutationen in den Gen-Loci ade 8-18 und ade 2-1 nicht in der Lage selbst Adenin zu synthetisieren. Daher benötigt dieser Stamm Adenin aus dem Außenmedium, um wachsen zu können. Dieses Adenin wird über den Purin/Cytosin-Transporter FCY2 der Hefe aufgenommen. Der Stamm DM734-284D ist jedoch in der Lage, auch auf Medium zu wachsen, welches statt Adenin Adenosin enthält, sofern ein Adenosin-Transport in die Hefezelle durch einen gentechnisch eingebrachten Adenosin-Transporter vermittelt wird. *Saccharomyces cerevisiae* selbst besitzt einen derartigen Adenosin-Transporter nicht. Daher eignet sich der Stamm DM734-284D als Komplementationssytem für die Isolierung von Adenosin-Transportern aus verschiedenen Organismen.

Um zu überprüfen, ob PUP1 den Transport von Adenosin vermittelt, wurde der Stamm DM734-284D mit den Konstrukten pDR195PUP1 und pDR195 als Kontrolle (Rentsch et al.,1995, *FEBS Lett.* 370, 264-368) nach Standardprotokoll zur Hefetransformation transformiert (Dohmen et al., 1991, *Yeast* 7, 691-692). Anschließend wurden zur Überprüfung der Transformation die Transformationsansätze auf Minimal-Medium plattiert,

welches 150 µM Adenin und die zum Wachstum erforderlichen Aminosäuren Arginin, Leucin, Tryptophan (je 60 mg/l) und Lysin (70 mg/l) enthielt. Als Transformationsmarker diente Uracil. Drei unabhängige positive Klone der Kontrolle und der PUP1 exprimierenden Klone wurden auf Minimal-Medium plattiert, welches statt 150 µM Adenin 150 µM Adenosin enthielt. Bei den PUP1-1 exprimierenden Klone zeigte sich nach drei Tagen deutliches Wachstum, während die mit dem Vektor pDR 195 transformierten Klone kein Wachstum zeigten (siehe Figur 8). Dies zeigt, daß PUP1 den Transport des Nucleosids und Adenin-Derivats Adenosin vermittelt.

Beispiel 6: PUP1-vermittelter Transport von Koffein in Hefe

Um zu überprüfen, ob PUP1 den Transport von Koffein vermittelt, wurde die Sensitivtät des *Saccharomyces cerevisiae*-Stammes MG877ura3$^-$ auf Koffein-haltigen Minimal-Medium ermittelt. Koffein wirkt ab bestimmten Konzentrationen auf Hefe toxisch (Bard et al., 1980, J. Bacteriol. 141, 999-1002), was zu langsameren Wachstum bzw. Tod der Hefen führt. Wenn PUP1 den Transport von Koffein vermittelt, so ist anzunehmen, daß ein Hefe-Stamm, welcher dieses Protein exprimiert, gegenüber Koffein eine höhere Sensitivität als der entsprechende Kontrollstamm besitzt, was sich in vermindertem Wachstum äussert.

Bei diesem Test wurden verschiedene Koffein-Konzentrationen zwischen 0 bis 1,5% Koffein im Minimal-Medium eingesetzt. 16 Stunden in flüssigem Minimalmedium gewachsene Hefe-Klone wurden auf den entsprechenden Platten ausgestrichen und für 6 Tage bei 28°C inkubiert. Dabei zeigte sich, daß PUP1 exprimierende Hefen gegenüber dem Kontrollstamm MG877ura3$^-$ und dem PUP2 exprimierenden Stamm ab der Konzentration von 0,2% Koffein ein deutlich verlangsamtes Wachstum aufwies (siehe Figur 9). Dies muß auf die erhöhte Aufnahme des toxischen Koffeins zurückgeführt werden und zeigt, daß PUP1 den Transport von Koffein vermittelt.

Patentansprüche

1. Nukleinsäure, die für einen pflanzlichen oder tierischen Nukleobasentransporter kodiert, ausgewählt aus:

a) Nukleinsäure, die erhältlich ist durch Komplementierung Nukleobasentransporter-defizienter Wirtszellen mit einer pflanzlichen oder tierischen Genbank und Selektion auf Nukleobasentransporter-positive Wirtszellen;

b) Nukleinsäure mit einer Sequenz, die für ein Protein mit einer Sequenz nach SEQ ID NO 8 oder SEQ ID NO 9 kodiert;

c) Nukleinsäure, die mit einer Nukleinsäure nach b) hybridisiert;

d) Nukleinsäure, die unter Berücksichtigung der Degeneration des genetischen Codes mit einer Nukleinsäure nach b) oder mit der zu b) komplementären Sequenz hybridisieren würde;

e) durch Substitution, Addition, Inversion und/oder Deletion einer oder mehrerer Basen erhaltene Derivate einer Nukleinsäure nach a) bis d);

f) Komplementäre Nukleinsäure zu einer Nukleinsäure nach einer der Gruppen a) bis e);

ausgenommen sind Nukleinsäuren mit einer Sequenz nach einer der SEQ ID NO 3 bis 5.

2. Nukleinsäure nach Anspruch 1, **dadurch gekennzeichnet,** daß sie die kodierende Sequenz einer der Sequenzen nach den SEQ ID NO 1, 2, 6, 7 oder 10 umfaßt oder ein von diesen durch Substitution, Addition, Inversion und/oder Deletion einer oder mehrerer Basen abgeleitetes Derivat.

3. Nukleinsäure nach einem der Ansprüche 1 oder 2, **dadurch gekennzeichnet,** daß sie eine DNA ist.

4. Fragment einer Nukleinsäure nach einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet,** daß es in Antisinnorientierung zu einem Promoter die Expression eines Nukleobasentransporters in einer Wirtszelle hemmen kann.

5. Fragment nach Anspruch 4, **dadurch gekennzeichnet,** daß es mindestens 10 Nukleotide, vorzugsweise mindestens 50 Nukleotide, besonders bevorzugt mindestens 200 Nukleotide umfaßt.

6. Konstrukt enthaltend eine Nukleinsäure nach einem der Ansprüche 1 bis 3 und/oder ein Fragment nach einem der Ansprüche 4 oder 5 unter der Kontrolle von die Expression regulierenden Elementen.

7. Konstrukt nach Anspruch 6, **dadurch gekennzeichnet,** daß sich die Nukleinsäure bzw. das Fragment in Antisinnorientierung zu dem regulatorischen Element befindet.

8. Konstrukt nach einem der Ansprüche 6 oder 7, **dadurch gekennzeichnet,** daß es in einem Plasmid vorliegt.

9. Wirtszelle enthaltend eine Nukleinsäure nach einem der Ansprüche 1 bis 3 und/oder eine Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder ein Fragment der vorgenannten Nukleinsäuren und/oder ein Konstrukt nach einem der Ansprüche 6 bis 8.

10. Wirtszelle nach Anspruch 9, **dadurch gekennzeichnet,** daß sie aus Bakterien, Hefezellen, Säugerzellen und Pflanzenzellen ausgewählt wird.

11. Transgene Pflanze sowie Pflanzenteile und/oder Samen der Pflanze enthaltend eine Nukleinsäure nach einem der Ansprüche 1 bis 3 und/oder eine Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder ein Fragment der vorgenannten Nukleinsäuren und/oder ein Konstrukt nach einem der Ansprüche 6 bis 8.

12. Transgene Pflanze, Pflanzenteil, Wirtszelle und/oder Samen nach einem der Ansprüche 9 bis 11, **dadurch gekennzeichnet,** daß die Nukleinsäure bzw. das Fragment bzw.

das Konstrukt an einer Stelle in das Genoms integriert ist, die nicht seiner natürlichen Position entspricht.

13. Protein erhältlich durch Expression einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 in einer Wirtszelle.

14. Antikörper, der mit einem Protein nach Anspruch 13 reagiert.

15. Verfahren zum Herstellen einer transgenen Pflanze, das die folgenden Schritte umfaßt:

- Einbringen einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder eines Fragments dieser Nukleinsäuren in eine Pflanzenzelle; und

- Regenerieren einer Pflanze aus der transformierten Pflanzenzelle.

16. Verfahren zum Beeinflussen der Nukleobasentransportereigenschaften einer Pflanze, eines Pflanzenteils, einer Pflanzenzelle und/oder von Samen, das den Schritt umfaßt:

- Einbringen einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 und/oder eines Fragments dieser Nukleinsäuren in eine Pflanzenzelle und/oder eine Pflanze.

17. Verwendung einer Pflanzenzelle nach Anspruch 10 zur Regeneration und Herstellung ganzer Pflanzen.

18. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 zur Isolierung homologer Sequenzen aus Bakterien, Pilzen, Pflanzen, Tieren und/oder Menschen.

19. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 zur Expression eines Nukleobasentransporters in pro- und/oder eukaryontischen Zellen.

20. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 unter der Kontrolle eines regulatorischen Elements in Antisinnorientierung zur Hemmung der Expression eines endogenen Nukleobasentransporters in pro- und/oder eukaryontischen Zellen.

21. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 zur Herstellung transgener Nutzpflanzen.

22. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 oder einer Nukleinsäure mit einer Sequenz nach einer der SEQ ID NO 3 bis 5 zur Identifizierung von Inhibitoren des Nukleobasentransports.

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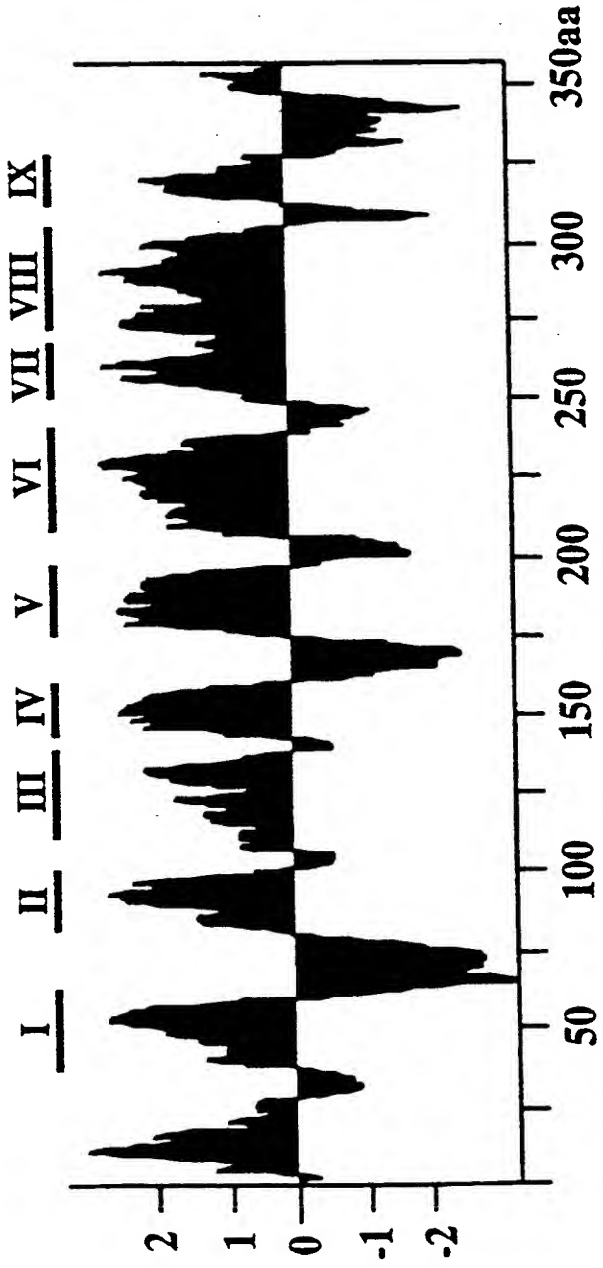

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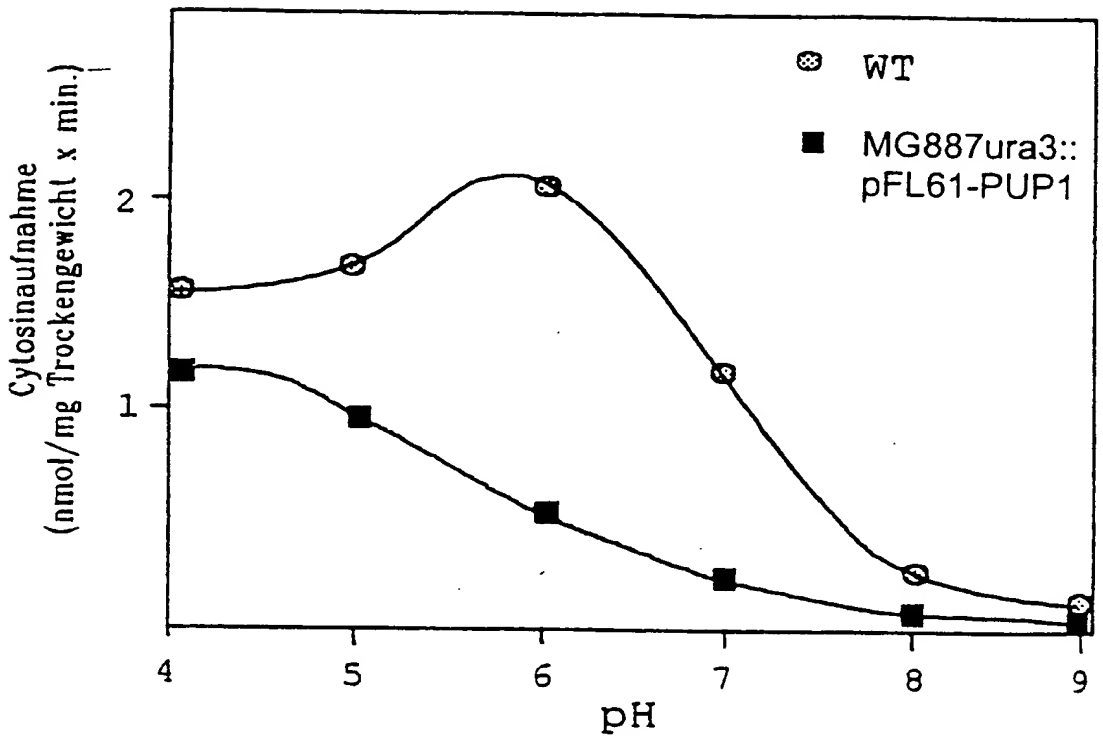

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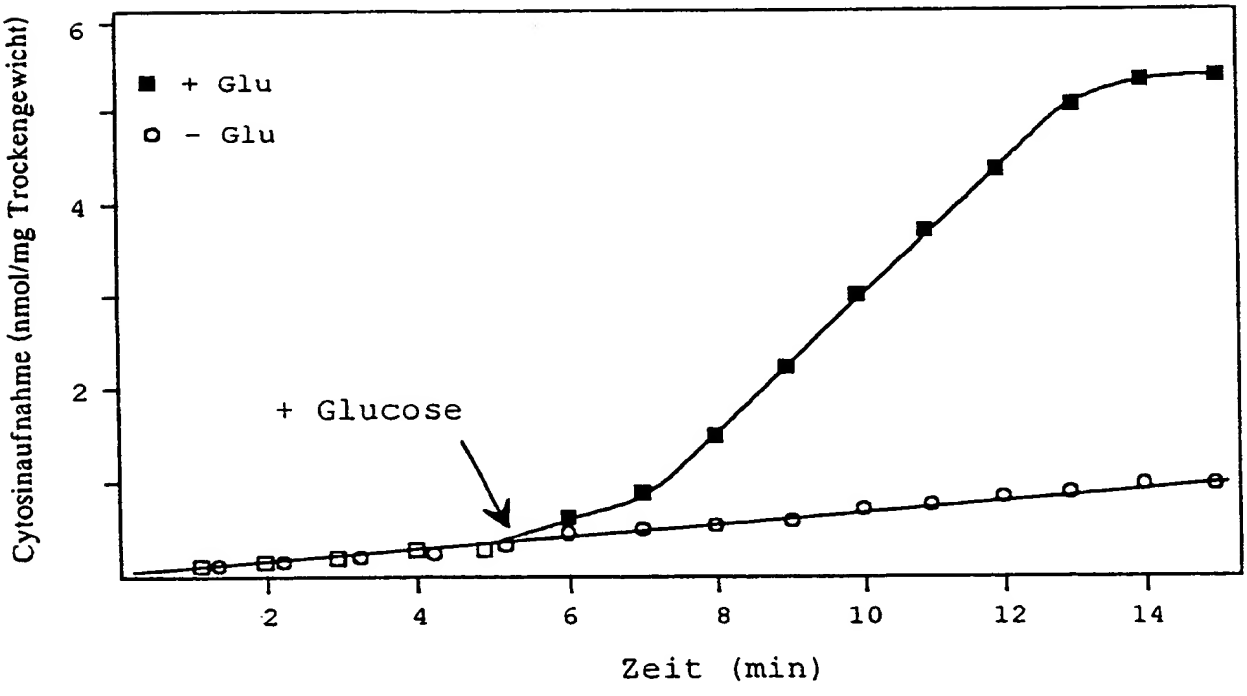

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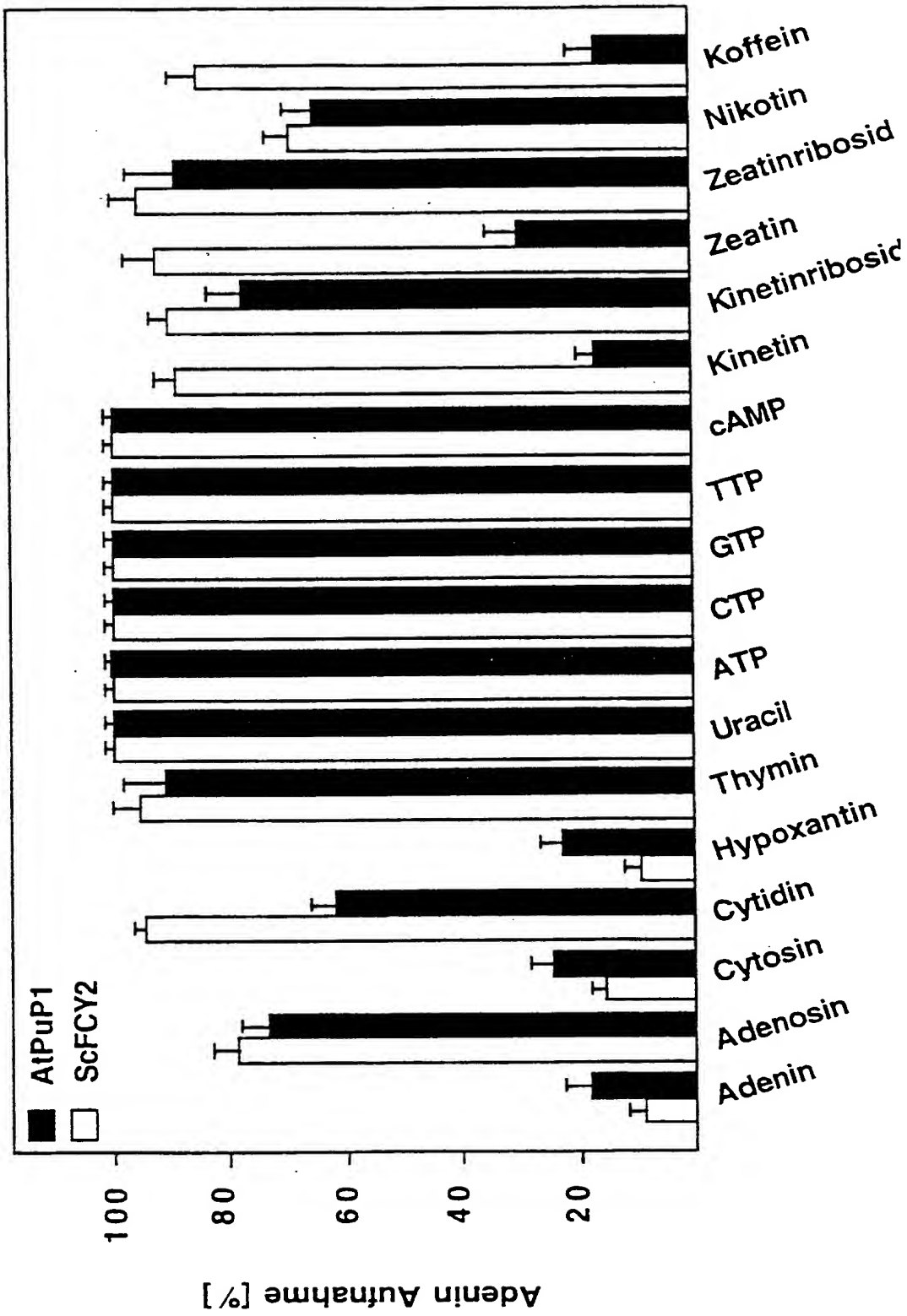
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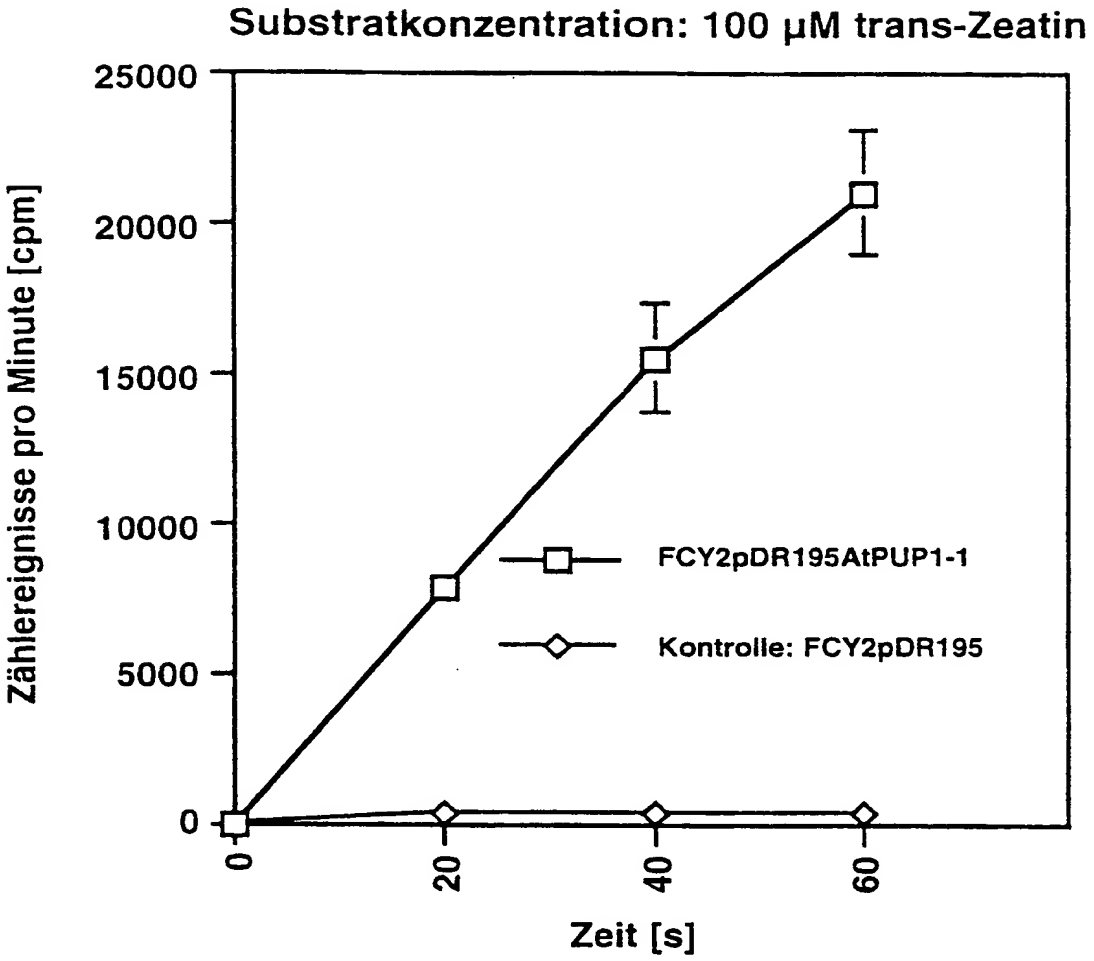
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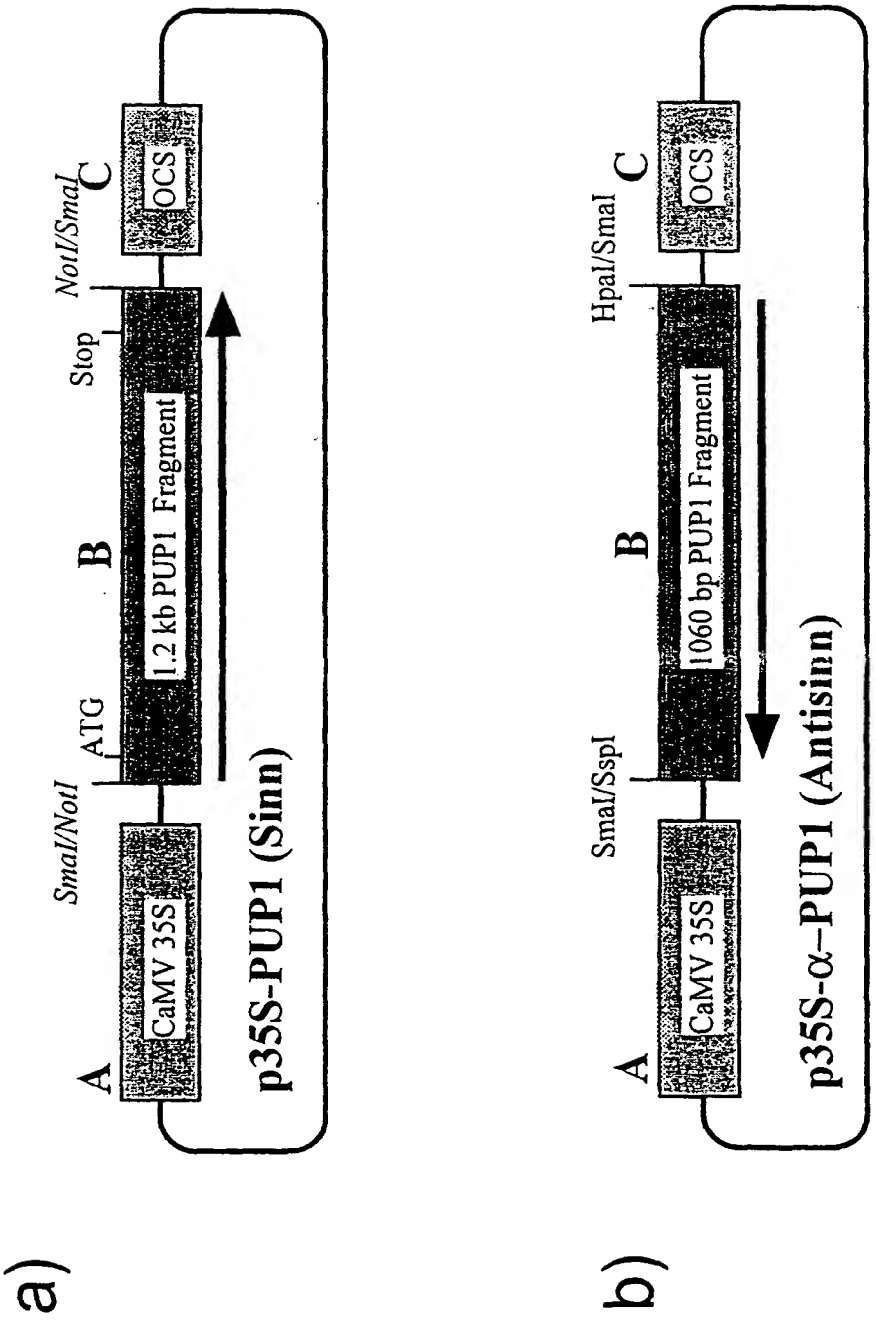
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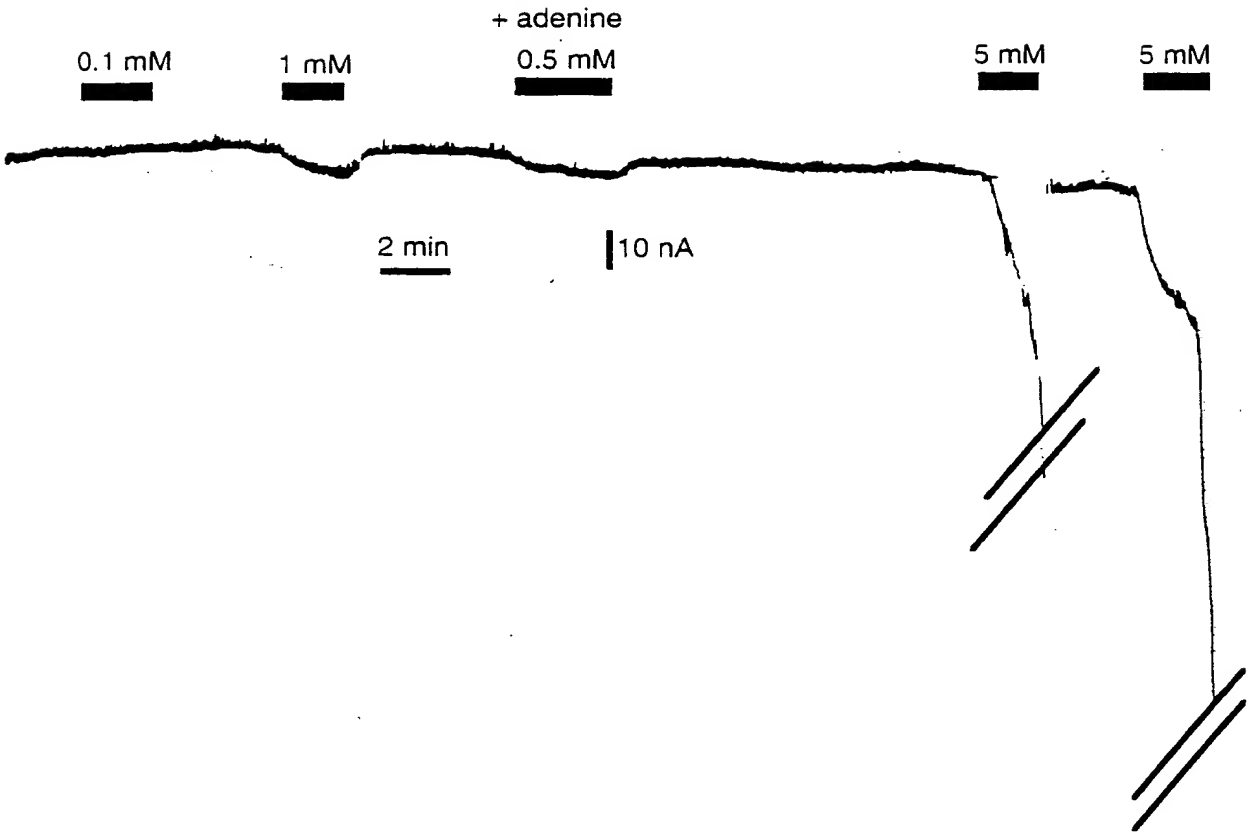

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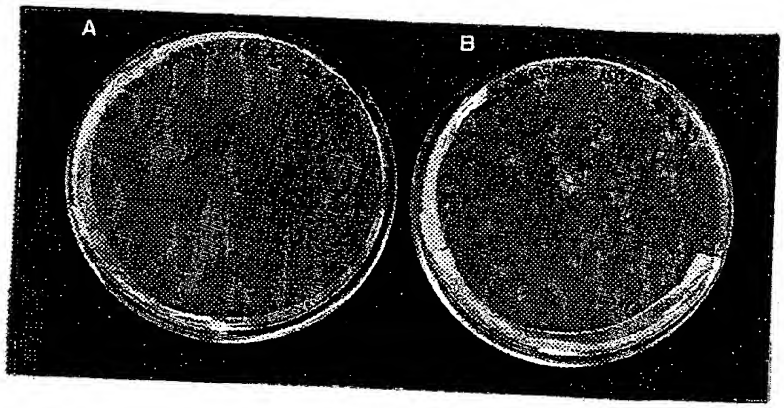

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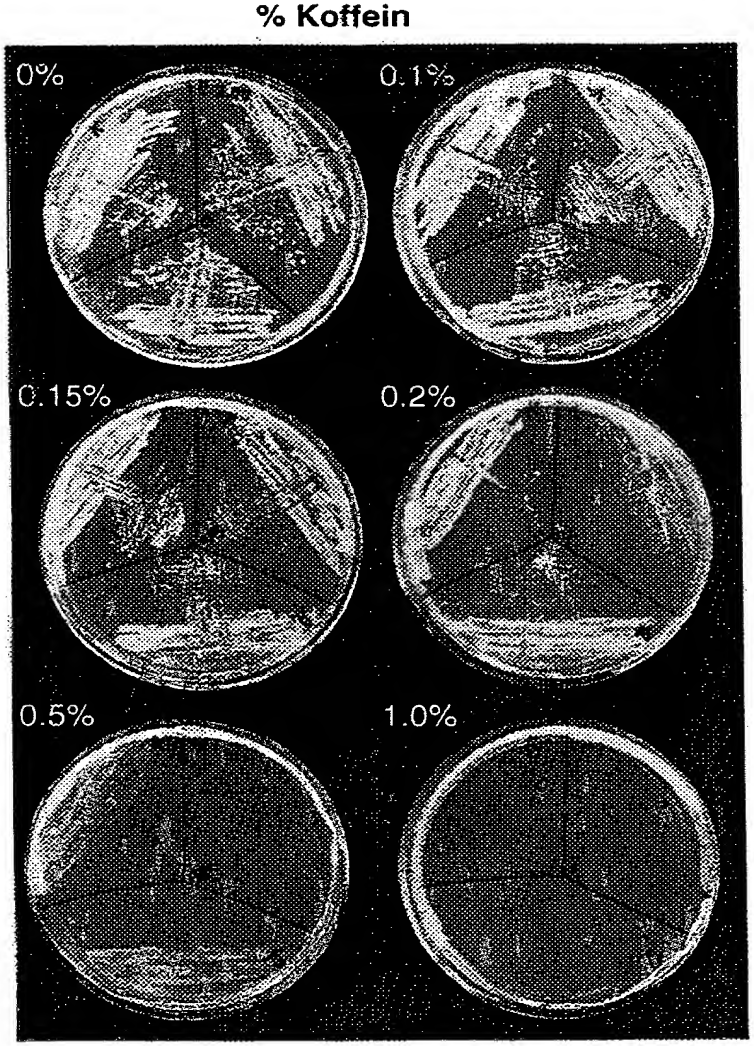

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p.d 01-1997
p.89-93 = (5)

Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs

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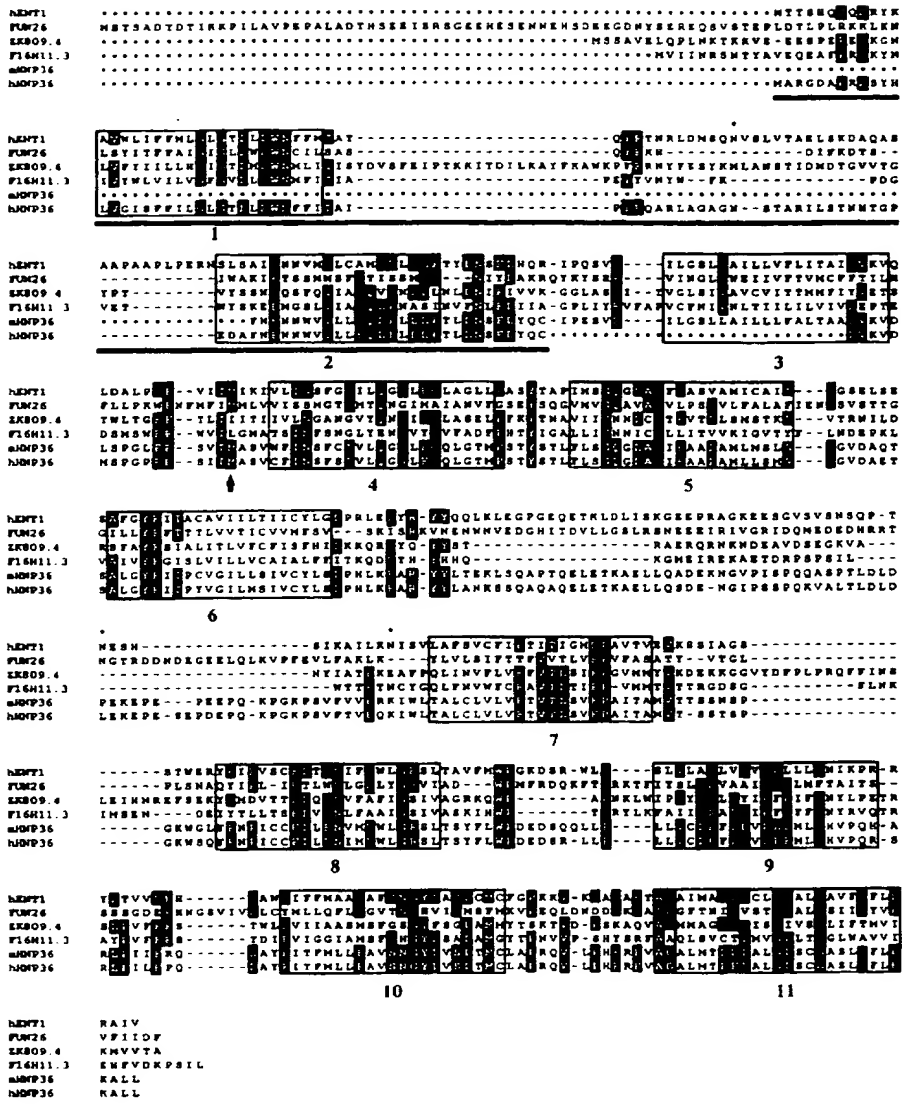
In most mammalian cells nucleoside uptake occurs primarily via broad-specificity, *es* (*e*, equilibrative; *s*, sensitive to NBMPR inhibition) transporters that are potently inhibited by nitrobenzylthioinosine (NBMPR)[1]. These transporters are essential for nucleotide synthesis by salvage pathways in hemopoietic and other cells that lack *de novo* pathways and are the route of cellular uptake for many cytotoxic nucleosides used in cancer and viral chemotherapy[1]. They play an important role in adenosine-mediated regulation of many physiological processes, including neurotransmission and platelet aggregation, and are a target for coronary vasodilator drugs. We have previously reported the purification of the prototypic *es* transporter from human erythrocytes[2] and have shown that this glycoprotein of apparent *M*, 55,000 is immunologically related to nucleoside transporters from several other species and tissues, including human placenta[3,4]. Here we report the isolation of a human placental cDNA encoding a 456-residue glycoprotein with functional characteristics typical of an *es*-type transporter. It is predicted to possess 11 membrane-spanning regions and is homologous to several proteins of unknown function in yeast, nematodes, plants and mammals. Because of its central role in the uptake both of adenosine and of chemotherapeutic nucleosides, study of this protein should not only provide insights into the physiological roles of nucleoside transport but also open the way to improved therapies.

Information derived from amino-terminal sequence analysis of the human erythrocyte *es* transporter was used to clone a human placental cDNA (GenBank accession no. U81375). The cDNA contains 178 and 616 base pairs (bp) of untranslated 5′ and 3′ regions, respectively, flanking a 1368-bp open reading frame (ORF) that encodes a 456-residue protein of *M*, 50,249, which we have designated hENT1 (human equilibrative nucleoside transporter 1) (Fig. 1*a*). hENT1 is predicted to contain 11 transmembrane (TM) segments connected by short (≤16 residue) hydrophilic regions, with the exception of the loops connecting TM 1 and 2, and TM 6 and 7, which are predicted to contain 41 and 66 residues, respectively. These large loops contain the three potential *N*-glycosylation sites in the protein. Our previous observation that the erythrocyte nucleoside transporter is glycosylated very close to one end of the protein[3] suggests that only the first of the three sites is glycosylated, and leads to the putative topography illustrated in Fig. 1*b*.

A search of the GenBank expressed sequence tag (EST) database revealed that hENT1 is expressed in adult heart, brain and mammary gland, and fetal liver and/or spleen, in addition to placenta and erythrocytes. hENT1 shows no significant sequence similarity to other known transport proteins, including the mammalian active nucleoside transporters cNT1 (ref. 6) and SPNT (ref. 7), and the bacterial nucleoside transporters nupC (ref. 8) and nupG (ref. 9). [AUTHOR, NOTE: WE DO NOT USE SUPERSCRIPTS ON NUMBERS OR ACRONYMS.] However, the predicted amino acid sequence is 23% and 21% identical, respectively, to ZK809.4 and F16H11.3, the predicted products of genes identified in the *Caenorhabditis elegans* genome sequencing project. It also exhibits 20% identity to the 517-residue, hypothetical protein FUN26 of *Saccharomyces cerevisiae*[10]. These three proteins, of unknown function, may correspond to nematode and yeast nucleoside transporters. The most highly conserved regions of the four sequences lie mainly within the putative transmembrane helices (Fig. 1*a*) and may therefore be structurally conserved because of involvement in substrate recognition and/or permeation. Database searching revealed a close homologue of one of these conserved regions (TM1) in an *Arabidopsis thaliana* EST (GenBank accession no. T20785), indicating the likely existence of related nucleoside transporters in plants. The carboxy-terminal two-thirds of the hENT1 sequence also shows strong similarity (approx. 44% identity) to the 36-kDa mouse and human HNP36 proteins (Fig. 1*a*). The latter are delayed-early response gene products, predicted to be shorter integral membrane proteins with eight membrane-spanning segments that evidently correspond to TMs 4–11 of hENT1. However, the 198 nucleotides upstream of the assigned start codon (arrow in Fig. 1*a*) in the mouse HNP36 cDNA are in frame with the rest of the coding sequence and could encode an amino acid sequence with 65% identity to the region of hENT1 containing TMs 2 and 3 (Fig. 1*a*), suggesting that the HNP36 cDNA may have been truncated at its 5′ end during preparation. Likewise, the nucleotide sequence upstream of the assigned start codon in the human HNP36 cDNA includes an ORF (underlined in Fig. 1*a*) that is 49% identical in predicted amino acid sequence to the N-terminal region (TMs 1 and 2) of hENT1. Both HNP36 proteins may there-



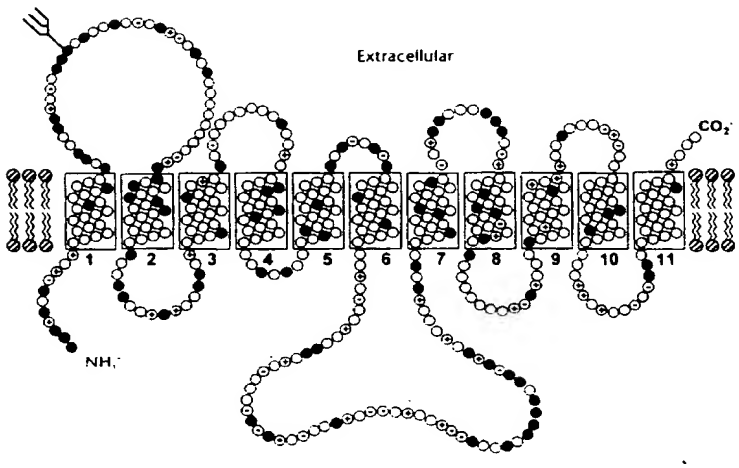


Fig. 1 *a*, Alignment of the predicted amino acid sequence of hENT1 with those of the yeast protein FUN26 (ref. 10), the nematode proteins ZK809.4 and F16H11.3, and the mouse (mHNP36) and human (hHNP36) delayed-early response gene proteins[11]. The positions of putative transmembrane regions are shown as open rectangles, and residues identical in four or more of the sequences are indicated by black boxes. The arrow shows the position of the residue previously assigned[11] as the N-terminus for the two HNP36 proteins, and the translated open reading frame upstream of this location in the human protein is underlined. Asterisks indicate potential *N*-linked glycosylation sites in hENT1. *b*, Topographical model of hENT1. Potential membrane-spanning α-helices are numbered and the putative *N*-glycosylation site at Asn48 is indicated. The positions of basic (Arg, Lys, His), acidic (Asp, Glu) and polar but uncharged residues (Ser, Thr, Gln, Asn) are indicated by ⊕, ⊖ and darkened circles (●), respectively.

fore be full-length homologues of hENT1 and possibly correspond to a second mammalian passive nucleoside transporter isoform. However, their function remains to be determined, given their reported nucleolar localization[11].

In order to investigate the functional properties of hENT1, the recombinant protein was expressed in *Xenopus* oocytes, which lack endogenous nucleoside transport activity[6,12]. The initial rate of uptake of [^{3}H]uridine (10 μM, 20 °C), which is only slowly metabolized by oocytes[5], was at least 30 times the rate in oocytes injected with hENT1 RNA transcript than in controls (Fig. 2*a*). Uptake was essentially linear for up to 5 minutes, and in subsequent experiments this period was used to approximate initial rates of transport. Values for 10 μM uridine influx (mean ± s.e.m., 8–10 oocytes) in nine independent experiments with different batches of oocytes ranged between 0.65 ± 0.04 and 1.61 ± 0.12 pmol/oocyte per 5 minutes in oocytes injected with hENT1 RNA transcript and between 0.009 ± 0.002 and 0.050 ± 0.006 pmol/oocyte per 5 minutes in control oocytes injected with water. Uridine uptake was not dependent on sodium ions and was unaffected by the presence of a high concentration (2 mM) of the nucleobase uracil, which is not a substrate for mammalian *es*-type nucleoside transporters[1] (Fig. 2*b*). However, uridine fluxes were strongly inhibited in the presence of 2 mM unlabeled uridine, thymidine, cytidine, adenosine, guanosine and inosine, a result that is consistent with the broad substrate specificity typical of *es* transporters[1] (Fig. 2*b*). Mediated influx of uridine, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable and conformed to simple Michaelis–Menten kinetics with an apparent K_m of 0.24 mM (Fig. 2*c*), which is within the reported range for uridine transport by mammalian *es* transporters[1]. The V_{max} for hENT1-mediated uridine influx was 18 pmol/oocyte per 5 minutes. Uridine

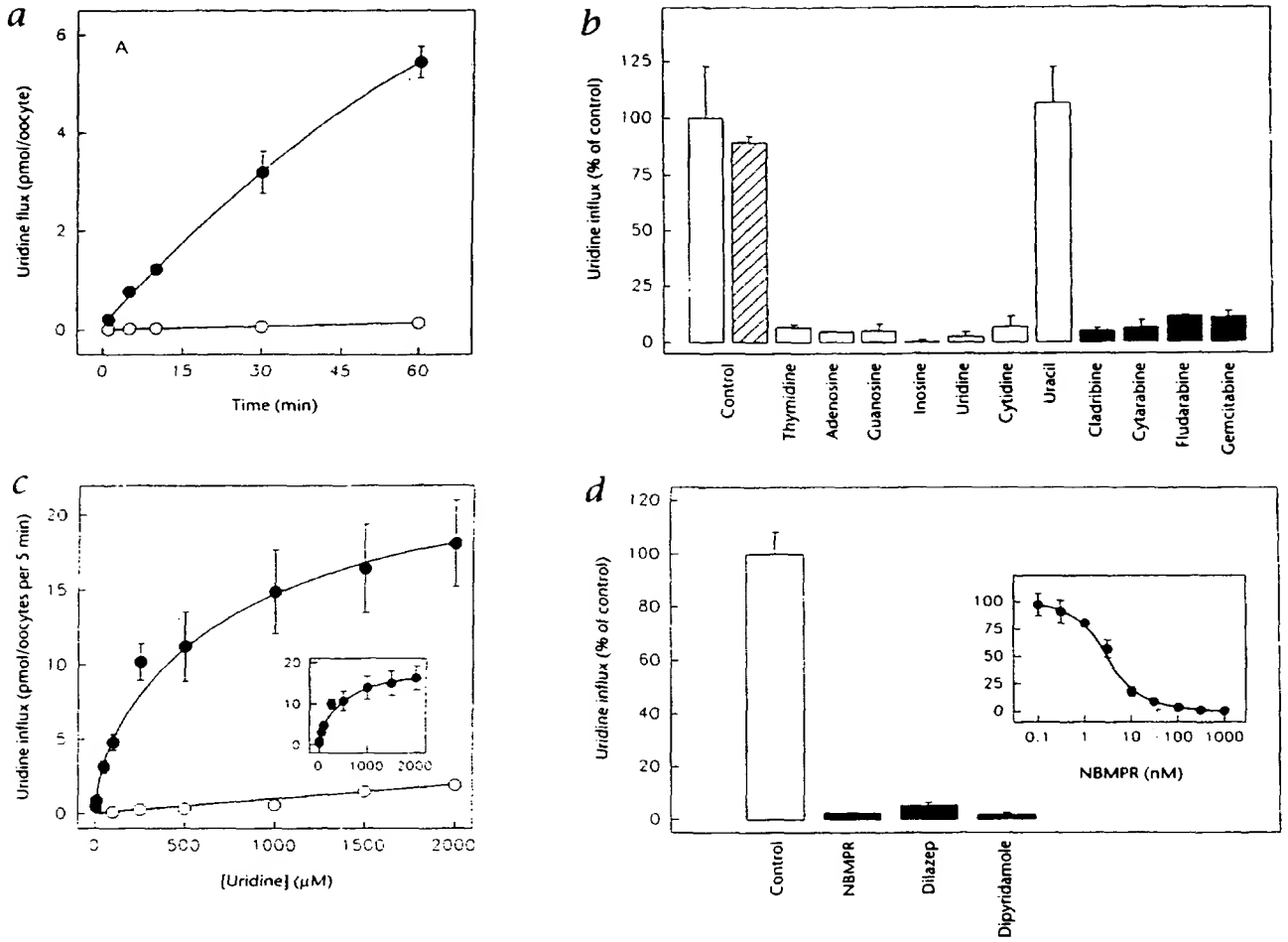


Fig. 2 *a*, Time-course of uridine uptake into *Xenopus* oocytes. Oocytes were injected with water alone (○) or water containing *in vitro* transcribed RNA from clone 17.1 (●). *b*, Specificity of clone 17.1-mediated uridine influx for physiological nucleosides and chemotherapeutic nucleoside analogues. The hatched column represents uptake measured in the absence of sodium. *c*, Concentration dependence of clone17.1-mediated uridine influx. Oocytes were injected with water alone (○) or water containing *in vitro* transcribed RNA from clone 17.1 (●). *inset*, Influx of uridine in RNA-injected oocytes minus that in water-injected oocytes. *d*, Inhibition of clone 17.1-mediated uridine influx by NBMPR, dilazep and dipyridamole. *inset*, The dose–response curve for NBMPR inhibition of uridine influx.

influx in water-injected oocytes exhibited a linear concentration dependence, consistent with passive diffusion of nucleoside across the oocyte plasma membrane[9,12] (Fig. 2*c*). Uptake of uridine (10 μM) by hENT1 was almost completely inhibited by NBMPR (1 μM) (Fig. 2*d*). The dose–response curve for NBMPR inhibition of hENT1-mediated uridine influx (Fig. 2*d*, inset) gave a calculated apparent K_i value of 2 nM, which is within the range of affinities reported for NBMPR binding to mammalian *es* transporters[1] (0.1–10 nM).

Equilibrative nucleoside transporters are pharmacologic targets for the coronary vasodilators dilazep and dipyridamole, which inhibit influx of adenosine across the plasma membrane and thereby potentiate the interaction of extracellular adenosine with purinoreceptors[1,13]. Both vasodilators potently inhibited uridine uptake by RNA-injected oocytes (Fig. 2*d*). Direct measurements of [^{14}C]adenosine (10 μM) fluxes in hENT1 RNA-injected and water-injected oocytes (Fig. 3*a*) gave a mediated flux of adenosine that was 3 to 4 times the corresponding flux for uridine (Fig. 2*a*). This adenosine flux showed the expected sensitivity to inhibition by uridine and NBMPR and was also strongly inhibited by dilazep and dipyridamole, indicating that hENT1 is likely to be a major target for these vasoactive drugs (Fig. 3*b*).

In order to investigate whether hENT1 has the capacity to mediate cellular uptake of anticancer nucleoside analogues, the effects on uridine influx by oocytes expressing the recombinant transporter were determined for cladribine[14], cytarabine[15] and fludarabine[16], three clinically important nucleoside drugs used for treatment of leukemias and lymphomas. A fourth nucleoside analogue, gemcitabine[17], which has recently been approved in several countries for the treatment of solid tumors, was also tested. In each case, the drugs, at a concentration of 2 mM, inhibited uridine uptake by ≥90% (Fig. 2*b*), suggesting that hENT1 is likely to be involved in the cellular uptake of these anticancer drugs.

Studies with photoaffinity labeling reagents, inhibitors and antibodies have revealed the existence of a family of related but functionally distinct *es*-type transporters in different mammalian tissues and species[1,4]. The rodent transporters are much less sensitive to inhibition by coronary vasodilators than are human ones, but are nonetheless recognized by anti-human transporter antibodies[1]. We have confirmed the sequence simi-

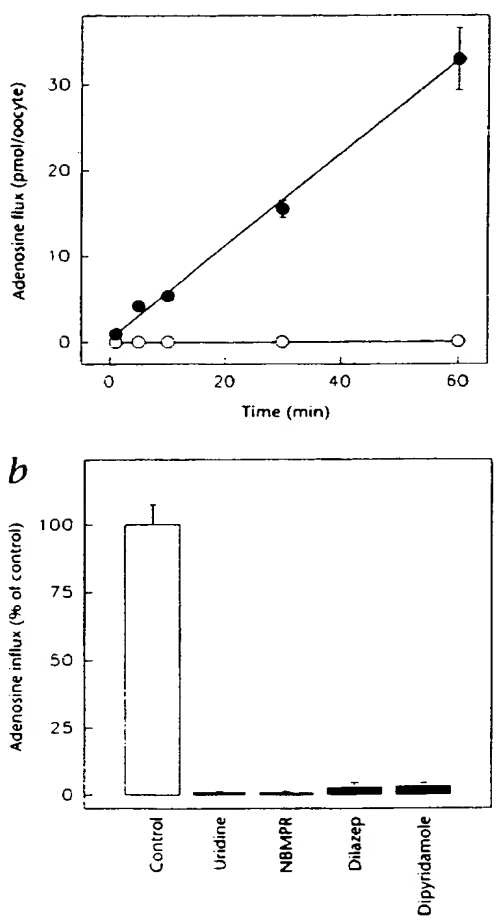


Fig. 3 *a*, Time course of adenosine uptake into *Xenopus* oocytes. Oocytes were injected with water alone (○) or with water containing *in vitro* transcribed RNA from clone 17.1 (●). *b*, Inhibition of clone 17.1-mediated adenosine influx by uridine, NBMPR, dilazep and dipyridamole.

larity of the rodent and human proteins by the identification, in the GenBank database, of ESTs encoding rat and mouse homologues of hENT1. This diversity of functional properties and tissue distributions has profound implications for chemotherapy, for example, relationships have been established between the levels of *es*-mediated transport and the anti-leukemic activity of nucleoside drugs[1]. The cloning of hENT1 now opens the way to the rapid identification of homologues and thus characterization of the structural features responsible for functional differences among transporters. Such knowledge should facilitate the design of novel nucleoside drugs with better targeting to diseased tissues and less toxicity to normal organs.

Methods

cDNA cloning and analysis. The sequence of the N-terminal 21 residues of the human erythrocyte nucleoside transporter, purified as previously described[2], was determined by gas-phase sequencing using an Applied Biosystems (Warrington, Cheshire, UK) model 477A sequencer to be TTSHQPQDRYKAV?LIFFMLG where "?" indicates an unidentified residue. A nondegenerate DNA sequence encoding residues 8 to 15 of this sequence was PCR-amplified using *Taq* polymerase from an oligo(dT)-primed human placental cDNA library constructed, using *Bst*XI linkers, in the plasmid vector pEF-BOS (ref. 18). The 5′ primer used for the amplification was an 8192-fold degenerate oligonucleotide corresponding to residues 1–7 plus an additional N-terminal methionine, whereas the 3′ primer was a 512-fold degenerate primer corresponding to residues 16–21. *Eco*RI and *Hin*dIII sites, respectively, were incorporated at the 5′ ends of the primers to facilitate subsequent cloning. A PCR product of the expected size (84 bp) was gel-purified, digested with *Eco*RI and *Hin*dIII and then ligated into the corresponding cloning sites of pBluescript II KS (+) (Stratagene, La Jolla, CA). Sequencing of the resultant clone (80A) showed that it encoded the amino acids expected for positions 8 to 15 in the N-terminal sequence of the transporter, and identified position 14 as tryptophan. Database searching showed the presence of an identical nucleotide sequence in a 265 bp EST (GenBank accession no. T25352) cloned from a human Burkitt's lymphoma cDNA library. This information was exploited to amplify the 3′ portion of the hENT1 cDNA by a nested PCR approach. Initial amplification of the placental library by touchdown PCR using Vent polymerase employed a 5′, nondegenerate primer based on the clone 80A sequence shown to encode residues 8–15, and a 3′ primer (pEF-BOS 2) corresponding to a region downstream of the *Bst*XI sites in pEF-BOS. The resultant products were subjected to a second amplification using Vent polymerase and a nested 5′ primer corresponding to bases 219–240 of the EST sequence. This yielded a single product of size 1.9 kb, which contained the complete coding sequence of the C-terminal region of the transporter, plus 616 bp of 3′ nontranslated region. The 5′ end of the transporter cDNA sequence was obtained by PCR amplification of the placental library by using as 3′ primer an oligonucleotide corresponding to nucleotides 1553–1572 of the sequence shown above and a 5′ primer (pEF-BOS 1) corresponding to a region of the pEF-BOS vector upstream of the *Bst*XI sites. The resultant 1.6 -kb product, which contained the complete coding sequence of the transporter plus 178 bp of untranslated 5′ sequence, was ligated into the *Sma*I site of pBluescript II KS (+) to yield clone 17.1, which was used for the expression experiments described in Figs. 2 and 3. At least two independent clones of each PCR product were sequenced and revealed no differences. All products were sequenced at least once on both strands by *Taq* DyeDeoxy terminator cycle sequencing using an Applied Biosystems model 373A DNA sequencer. Assignment of the translation initiation site of the ORF in hENT1 cDNA was based on its resemblance to the consensus sequence described by Kozak[19], and exact correspondence between the deduced N-terminal sequence and that determined for the erythrocyte protein, except that the latter lacks the initial methionine residue. Analysis of the protein sequence for the presence of putative membrane-spanning segments (Fig. 1) was performed by the method of Hofmann and Stoffel[20].

***Xenopus* expression and nucleoside uptake assays.** Plasmid DNA was linearized with *Not*I and transcribed with T3 polymerase in the presence of ^{m7}GpppG cap using the Megascript (Ambion, Austin, TX) transcription system. Remaining template was removed by digestion with RNase-free DNase 1. Oocytes were treated with collagenase to remove follicular layers[12] and then injected with 10 ng clone 17.1 cRNA in 10 nl of water or 10 nl of water alone. After 3 days, uptake of [5,6-^{3}H]uridine (2 Ci/mmol, HPLC-purified before use; Moravek Biochemicals, Brea, CA) (Fig. 2) or 10 μM [^{14}C]adenosine (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) (Fig. 3) was performed at 20 °C on groups of 10–12 oocytes in transport buffer (0.2 ml) containing 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5. In adenosine uptake and competition experiments, the transport buffer contained 1 μM deoxycoformycin to inhibit adenosine deaminase activity. At this concentration, deoxycoformycin had no effect on uridine influx. Except where otherwise indicated the incubation period was 5 min and the permeant concentration was 10 μM. Competing unlabeled nucleosides or uracil were used at a concentration of 2 mM. For experiments involving NBMPR, dilazep and dipyridamole, oocytes were treated for 1 h with inhibitor (1 μM unless otherwise indicated) before the addition of permeant. At the end of the incubation, extracellular label was removed by six rapid washes with ice-cold transport buffer. Individual oocytes were dissolved in 5% SDS for quantification of radioactivity by liquid scintillation counting. Each of the values shown represents the mean ± s.e.m. of 8–10 oocytes. Fluxes shown in Fig. 2, *b* and *d*, and Fig. 3*b* were corrected for endogenous nucleoside uptake activity by subtraction of fluxes seen in water-injected oocytes. This flux was not affected by the presence of competing nucleosides or inhibitors in the transport buffer. Apparent K_m (0.24 ± 0.03 mM) and V_{max} (18 ± 1 pmol/oocyte per 5 min) values for uridine influx (Fig. 2*c*) were determined by nonlinear re-

gression analysis (Enzfitter, Elsevier-Biosoft, Cambridge, UK). Linear regression analysis of an indirect Hill plot transformation of the dose–response curve for NBMPR inhibition of hENT1-mediated uridine influx (Fig. 2*d*) gave a Hill coefficient of 0.98 ± 0.03 (indicating interaction of NBMPR with a single population of binding sites) and an IC_{50} value of 3.4 ± 0.03 nM. Correction of the latter value for inhibitor depletion caused by partitioning into oocyte lipids (determined using HPLC-purified [^{3}H]NBMPR (Moravek Biochemicals) to be 26%) gave an apparent K_i value for NBMPR inhibition of uridine influx of 2.4 nM (calculated using competitive inhibition[11]) and a uridine K_m of 0.24 mM). Gemcitabine was a generous gift from Eli Lilly Company.

Acknowledgments

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Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase

PHILIP R. KUHL & LINDA G. GRIFFITH-CIMA

Nature Medicine **2**, 1022–1027 (1996)

On page 1025 of the September issue, Figure 4 was incorrect. The correct version is displayed below.

Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy

A. LAROCHELLE, J. VORMOOR, H. HANENBERG, J.C.Y. WANG, M. BHATIA, T. LAPIDOT, T. MORITA, B. MURDOCH, X.L. XIAO, I. KATO, D.A. WILLIAMS & J.E. DICK

Nature Medicine **2**, 1329–1337 (1996)

The abstract incorrectly stated that the SCID-repopulating cells were exclusively present in the $CD4^+CD8^-$ fraction; they were exclusively in the $CD34^+CD38^-$ fraction. We regret the error.

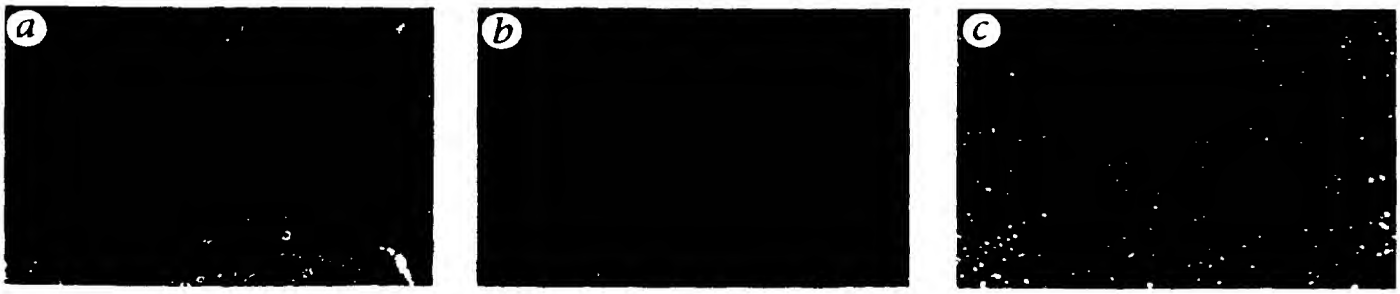

Fig. 4 Attenuation of cell spreading by small PEO stars and soluble or tethered EGF. Morphology of cells (×10 magnification, phase contrast) after 1 day in culture. Under all conditions, cell morphologies were invariant during the culture period after initial spreading had occurred. Two-thirds of the medium was replaced daily. *a*, Moderate inhibition of spreading of hepatocytes cultured on aminated glass slides grafted with star PEO (f = 70, M_r = 5200) from a 1% solution; *b*, complete inhibition of hepatocyte spreading by the addition of 10 ng/ml soluble EGF to hepatocytes cultured on slides grafted with star PEO (f = 70, M_r = 5200) from a 1% solution; *c*, complete inhibition of hepatocyte spreading by 4 ng/cm^2 tethered EGF, where EGF is linked to star PEO (f = 70, M_r = 5200) grafted to the surface from a 1% solution.

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P. 663-665

Cloning and Expression in Yeast of a Plant Potassium Ion Transport System

Hervé Sentenac, Nathalie Bonneaud, Michèle Minet, François Lacroute, Jean-Michel Salmon, Frédéric Gaymard, Claude Grignon

A membrane polypeptide involved in K^+ transport in a higher plant was cloned by complementation of a yeast mutant defective in K^+ uptake with a complementary DNA library from *Arabidopsis thaliana*. A 2.65-kilobase complementary DNA conferred ability to grow on media with K^+ concentration in the micromolar range and to absorb K^+ (or $^{86}Rb^+$) at rates similar to those in wild-type yeast. The predicted amino acid sequence (838 amino acids) has three domains: a channel-forming region homologous to animal K^+ channels, a cyclic nucleotide-binding site, and an ankyrin-like region.

In contrast to animal cells, plant cells are generally exposed to low K^+ concentrations, often in the micromolar range. Growth in such conditions is made possible by high affinity K^+ transport systems in the plasma membrane (*1*). Biochemical efforts to purify the transporters are difficult, because of the low abundance of these transport proteins, and screening cDNA libraries with heterologous DNA probes has been generally unsuccessful in plants (*2*).

A mutant (*3*) of *Saccharomyces cerevisiae*, unable to grow on low K^+ medium and belonging to the same complementation group as the *TRK1* (*4*) K^+ transport system, was here complemented with a cDNA library made from *Arabidopsis thaliana* seedlings. An *Arabidopsis* clone (AKT1) was able to complement the yeast mutant and effect K^+ transport (Fig. 1). In the low (micromolar) K^+ concentration range, the K^+ (or $^{86}Rb^+$) uptake rates were similar in the wild-type and complemented yeast strains; both were much higher than in the mutant strain. When the K^+ concentration was increased to the millimolar range, the uptake rate reached a saturation plateau in the wild-type strain but in the complemented strain continued to increase with increasing K^+ concentration. The kinetics of the K^+ transport in the complemented strain were complex (Fig. 1B) and did not fit classical (Michaelian) saturation kinetics. The kinetics of K^+ uptake in plant roots are also quite complex (*1*) and may nevertheless result from the activity of a single transport system (*5*).

The capacity of the protein encoded by AKT1 to accumulate K^+ was verified by transferring complemented yeast into a K^+-free medium. After an initial loss of K^+ (*3*), which increased the external K^+ concentration to 10 μM, a net influx developed, decreasing the external K^+ concentration to 0.65 μM (*6*). Under these conditions, the cytosolic K^+ concentration was estimated to be 0.17 M (*7*). Thus, yeast transformed with AKT1 maintained a high K^+ accumulation ratio (K_{int}/K_{ext} approximately 2.6×10^5), which corresponded to an equilibrium potential difference (E_K) of about −320 mV. No estimate of the actual membrane potential difference is available, and it was not possible to determine whether the protein encoded by AKT1 mediated passive or active K^+ transport (*8*).

A Southern (DNA) blot of Eco RI–digested genomic DNA from *Arabidopsis* probed with the 2.65-kb AKT1 cDNA showed a single band. Northern (RNA) blot of total RNA from *Arabidopsis* indicated that a single 2.8-kb transcript hybridized with the AKT1 cDNA. The difference in length between the transcript and the cDNA may result from the loss of the polyadenylate tail or from cloning a cDNA incomplete in the 5′ upstream region (Fig. 2).

The AKT1 cDNA encodes a predicted peptide of 838 amino acids (MW 95.4 kD) (Fig. 2). No homology was found with the yeast TRK1 gene product (*4*) or with K^+-transporting ATPases from bacteria and

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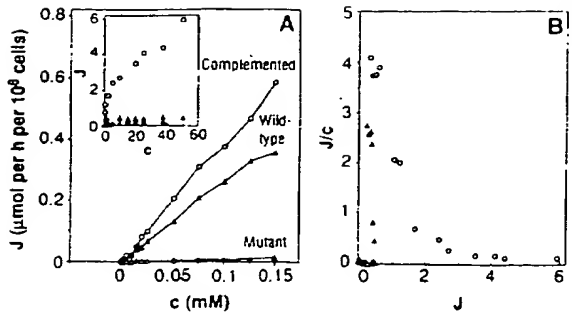

Fig. 1. Heterologous expression of an *A. thaliana* K^+ channel in *S. cerevisiae* (**A**) $^{86}Rb^+$ influx (J), in media of various K^+ concentrations (c). (**B**) Eadie-Hofstee transformation of the data shown in (A). A double mutant defective in K^+ uptake and deleted for the *URA3* gene was obtained from a cross between the PC1 strain (*3*) and a *URA3Δ* strain. This double mutant was complemented by the spheroplast transformation method with an *Arabidopsis* cDNA library and the shuttle vector pFL61 (*30*) bearing the yeast *URA3* marker. Expression of the cDNA was controlled by the constitutive promotor of the phosphoglycerate kinase gene. The transformants were grown on a solid medium containing 20 μM K^+. Twelve independent clones with growth characteristics similar to the wild-type strain were obtained from 6×10^5 transformants. The corresponding plasmids were subcloned in *E. coli* and reintroduced into the mutant yeast strain, which they again stably complemented. K^+ transport was studied (*31*) with the mutant strain complemented by one of the plasmids (pHS41).

Fig. 2. Deduced amino acid (*20*) sequence encoded by AKT1 cDNA in plasmid pHS41. The nucleotide sequence of the 2649-bp cDNA revealed a single 2517-bp open reading frame (nucleotides 58 through 2574). The cloned cDNA did not have a polyadenylate tail. The putative transmembrane segments (S1 through S6) and the H5 region in the predicted peptide are underlined. The EMBL accession number of the nucleotide and amino acid sequences is X62907.

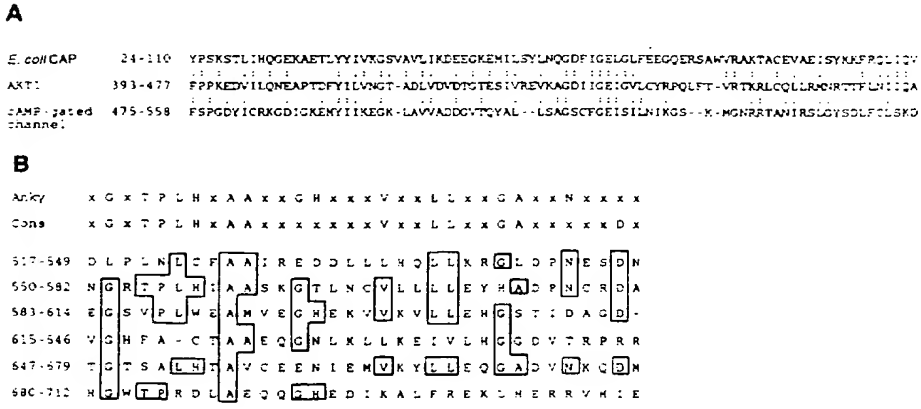

Fig. 3. Analysis of the AKT1 polypeptide sequence. (**A**) Homologies with putative cyclic nucleotide–binding sites. The displayed region was identified as a putative cyclic nucleotide–binding site by comparison with *E. coli* CAP (*24*) and cyclic nucleotide–gated channels (*10, 11*). (**B**) Comparison of aligned ankyrin-like repeats of AKT1 with the erythrocyte ankyrin consensus (*26*) (Anky), and with the consensus (Cons) for similar repeats in nine other proteins (*28*). An "x" in the Anky and Cons sequences marks a position where no consensus exists. Amino acid gaps (marked by hyphens) were introduced for maximal alignments (*13*). Identical residues are indicated by colons or by boxes, and conservative substitutions by single points. Numbers: location of the first and last amino acids of each repeat. Amino acid groups for conservative changes: [K, R]; [D, E]; [N, Q]; [A, G]; [S, T]; [I, M, L, V]; [F, W, Y].

mammals (9). However, homologies wit cAMP-gated channels from rat or bovin olfactory epithelium (*10, 11*) extend from amino acids 36 to 522 [22.3 or 22.5% identity within that region]. Homologies with the cGMP-gated channel from bovine rods (*12*) extend from amino acids 49 t 507 (22.7% identity).

Homologies were found (*13*) with K channels of the Shaker family found insects and mammals (*14*). The Shak channels are thought to consist of fou subunits (*15*) arranged about a central pore; each subunit consists of six transmembrane segments called S1 to S6 (*16*). Site-directed mutagenesis and electrophysiological measurements suggest that S4 acts as a voltage sensor (*17*). Th sequence of S4 is characterized by repet tion of basic amino acids at every third fourth position (*14*). In the protein enco ed by AKT1, a region homologous to S4 i present between amino acids 160 and 18 that has six basic residues conserved out of the seven present in the corresponding region of the Shaker K^+ channel ShB (*14, 17*).

Among K^+ channels, the highest degre of sequence identity is found in the putativ pore-forming sequence (*18*), called H (*14*), located between S5 and S6 a thought to span the membrane as a hairpin. Amino acids 242 to 266 of th protein encoded by AKT1 show homolog with the H5 region (*14*) of voltage- or cyclic nucleotide–gated K^+ channels. The ends of the H5 segment are parts of the external vestibule of the channel (*19*), and the amino acid motif T-T-V (*20*) within H5 (corresponding to amino acids 252 t 254 in the protein encoded by AKT1) a involved in the formation of the intern vestibule (*18*). In the Shaker K^+ channe ShB, amino acids 433 (external vestibule), 441, and 442 control the cation selectivity (*21*). In the protein encoded by AKT1, the F at position 433 is conservatively replaced

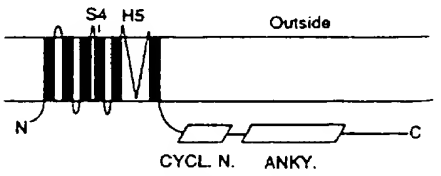

Fig. 4. Proposed topography of the AKT1 polypeptide. The averaged hydropathicity index values (*32*) were calculated with a window size of 11 amino acids. We assumed that the ankyrin-related domain (ANKY), the putative cyclic nucleotide–binding site (CYCL. N.), and the middle portion of H5 (*18*) were intracellular and that the ends of H5 were extracellular (*19*). The six transmembrane segments, including S4 (*17*), are shaded. N, NH_2-terminus; C, COOH terminus.

by a Y at 245, and the Ts at 441 and 442 are conserved (252 and 253).

The region between amino acids 393 and 477 is homologous with mammalian ion channels (*10–12*) and protein kinases (*22*) that are regulated by cGMP or cAMP, and with the catabolite gene activator protein (CAP) of *Escherichia coli* (*23*) (Fig. 3A), which includes a cAMP binding site (*24*). Similarly, the corresponding regions of cyclic nucleotide–gated channels are thought to bind cAMP (*10, 11*). The significance of homologies to these regions in the protein encoded by AKT1 is not clear because there is as yet no conclusive evidence for the presence of cyclic nucleotides in higher plants (*25*).

The six imperfect repeating sequences of 32 or 33 amino acids between positions 517 and 712 of the protein encoded by AKT1 (Fig. 3B) show homology to a 33-residue motif repeated 22 times in tandem in erythrocyte ankyrin (*26*), a protein that attaches integral membrane proteins to cytoskeleton components. In brain, ankyrin links a voltage-dependent Na^+ channel to spectrin, and thus may restrict the channel to specific locations in the neuronal membrane (*27*). Similar repeats have been observed in a variety of proteins and are thought to tether the subunits of regulatory proteins (*28, 29*). Thus, the presence of ankyrin-like repeats in the protein encoded by AKT1 suggests that this transport system may interact with the cytoskeleton or with regulatory proteins.

The sequence homologies between the protein encoded by AKT1 and cyclic nucleotide–gated channels encompass the six putative transmembrane segments present in the channels between the NH_2-terminus and the cyclic nucleotide–binding region. These homologies with this region of the channels suggest that the protein encoded by AKT1 also has six transmembrane segments (Fig. 4), although only four (S1, S2, S5, and S6) may be inferred from the hydropathicity plot. Whatever the exact topography, the presence in a plant transport system of both the highly conserved S4 and H5 regions, typical of Shaker channels, and of a cyclic nucleotide–binding site supports the hypothesis of an ancient common origin of voltage-gated and cyclic nucleotide–gated channels (*14*).

It is unlikely that the protein encoded by AKT1 is an accessory polypeptide interacting with yeast transport proteins, because the protein encoded by AKT1 was not homologous to the yeast *TRK1* gene product (*4*), and expression of AKT1 in the mutant yeast strain was sufficient to form a functional K^+ uptake system. The homologous Shaker polypeptides form functional channels when expressed in *Xenopus* oocytes (*15*).

Note added in proof: Functional expression of another putative K^+ transport system cDNA (*KAT1*) from *Arabidopsis thaliana* in a yeast *trk1*Δ *trk2*Δ mutant has just been reported (*29a*). The predicted amino acid sequences of AKT1 and KAT1 share extensive identity but are not allelic.

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31. Yeast cells were grown to early stationary phase in a synthetic liquid (*3*), incubated for 3 hours at 28°C, centrifuged, and resuspended at 2.5×10^{10} cells per milliliter. A 20-μl aliquot of the yeast suspension was mixed with 20 μl of a medium (*3*) containing KCl and $^{86}Rb^+$ (12 KBq per ml). After 3 minutes, uptake was stopped by addition of 10 ml of ice-cold 2 mM $CaSO_4$. The cells were collected on filters, washed with 10 ml of ice-cold $CaSO_4$, dried, and assayed for radioactivity. Uptake was linear with time for the first 5 minutes of incubation.
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33. We thank A. Rodriguez-Navarro for the PC1 mutant, S. Liang for the *URA3*Δ strain, and M. Lepetit, D. T. Clarkson, and S. Staunton for advice and comments on the manuscript.

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DNA Hydrolyzing Autoantibodies

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A DNA-nicking activity was detected in the sera of patients with various autoimmune pathologies and was shown to be a property of autoantibodies. The DNA hydrolyzing activity, which was purified by affinity and high-performance liquid chromatography, corresponded in size to immunoglobulin M (IgM) and IgG and had a positive response to antibodies to human IgG. The DNA hydrolyzing autoantibodies were stable to acid shock and yielded a DNA degradation pattern that was different from that of deoxyribonuclease (DNase) I and blood DNase.

Patients with autoimmune diseases produce autoantibodies to nucleoprotein complexes (*1*), to DNA, and to enzymes that participate in nucleic acid metabolism (*2*). In autoimmune diseases, there can be spontaneous induction of anti-idiotypic antibodies (Abs), which are Abs elicited by a primary antigen. These anti-idiotypic Abs may have characteristics of the primary antigen, including catalytic activity. In some cases, the sera of patients with scleroderma, systemic lupus erythematosus (SLE), or rheumatoid arthritis have an

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A New Family of High-Affinity Transporters for Adenine, Cytosine, and Purine Derivatives in Arabidopsis

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In many organisms, including plants, nucleic acid bases and derivatives such as caffeine are transported across the plasma membrane. Cytokinins, important hormones structurally related to adenine, are produced mainly in root apices, from where they are translocated to shoots to control a multitude of physiological processes. Complementation of a yeast mutant deficient in adenine uptake (*fcy2*) with an Arabidopsis cDNA expression library enabled the identification of a gene, *AtPUP1* (for *Arabidopsis thaliana* purine permease1), belonging to a large gene family (*AtPUP1* to *AtPUP15*) encoding a new class of small, integral membrane proteins. AtPUP1 transports adenine and cytosine with high affinity. Uptake is energy dependent, occurs against a concentration gradient, and is sensitive to protonophores, potentially indicating secondary active transport. Competition studies show that purine derivatives (e.g., hypoxanthine), phytohormones (e.g., zeatin and kinetin), and alkaloids (e.g., caffeine) are potent inhibitors of adenine and cytosine uptake. Inhibition by cytokinins is competitive (competitive inhibition constant K_i = 20 to 35 μM), indicating that cytokinins are transported by this system. *AtPUP1* is expressed in all organs except roots, indicating that the gene encodes an uptake system for root-derived nucleic acid base derivatives in shoots or that it exports nucleic acid base analogs from shoots by way of the phloem. The other family members may have different affinities for nucleic acid bases, perhaps functioning as transporters for nucleosides, nucleotides, and their derivatives.

INTRODUCTION

Nucleic acid bases are essential for a wide spectrum of metabolic processes, not the least of which is nucleic acid synthesis. Derivatives of nucleic acid bases and nucleotides play potentially important roles in energization, cell division, senescence, and defense reactions. Alkaloids, such as theobromine, caffeine, and nicotine, are structurally closely related to nucleic acid bases. Other important purine derivatives are cytokinins, which serve as hormones that control many processes in the plant (Chen et al., 1985; Chen, 1997).

Many examples of nucleic acid base and nucleoside uptake in plants are known, but the respective transporter genes have not been identified. Specific transport systems for uracil and guanine have been described in *Chlorella fusca* (Knutsen, 1972; Pettersen and Knutsen, 1974). Uptake of adenine by cell cultures of *Acer pseudoplantanus* and of uridine by *Lemna gibba* have also been demonstrated (Doreé, 1973; Nakashima and Tsuzuki, 1976).

Adenosine, guanosine, cytidine, and uridine are taken up against a concentration gradient into petunia pollen (Kamboj and Jackson, 1984, 1985, 1987). In contrast, uptake of thymidine in the same system occurs by facilitated diffusion at much lower rates. These data are consistent with a role for nucleosides in germinating pollen—mainly in RNA synthesis and DNA repair—which is consistent with the supply of nucleoside precursors from the carpel after pollen germination (van der Donk, 1974; Jackson and Linskens, 1978, 1980).

During germination, storage reserves from the endosperm are metabolized and translocated into the developing seedling. Besides secretion of sucrose and amino acids, adenine, adenosine, and guanosine are also exported from isolated endosperm tissue into the medium. *Ricinus communis* cotyledons separated from the endosperm take up endosperm-derived secretion products, including purine and pyrimidine bases, nucleosides, and AMP, with high efficiency, but not ATP (Kombrink and Beevers, 1983). Uptake of adenine may also play an important role for ATP synthesis. In seeds, ATP accumulates soon after hydration, a result of the conversion of adenine to AMP by adenine phosphoribosyltransferase (Moreland et al., 1974; Lee and Moffat, 1994). Exogenous adenine is readily taken up and converted into AMP and ATP (Lee and Moffat, 1994). Thus, efficient

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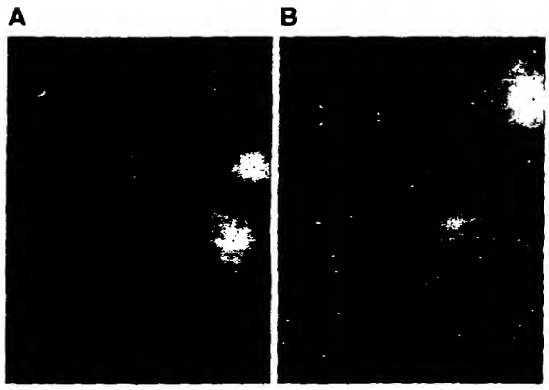

Figure 1. Functional Complementation of MG887-1 by *AtPUP1* (in pDR195).

Growth was on minimal medium containing 7.4 mM adenine as the sole nitrogen source.
(A) MG887-1pDR195 is the *fcy2* mutant transformed with pDR195, which served as the control.
(B) MG887-1pAtPUP1 is the *fcy2* mutant transformed with *AtPUP1* in pDR195.

adenine uptake from the endosperm may be essential for supplying the germinating seedling with sufficient ATP during early stages of development.

Purine-related alkaloids, such as caffeine, are translocated in the plant and found as constituents of xylem sap (Mazzafera and Gonçalves, 1999). Also, as shown by grafting experiments, nicotine is produced in tobacco roots and then is transported to leaves (Dawson, 1942). Therefore, transport systems for alkaloids must be present in plants.

External application of cytokinins leads to turnover inside plant cells, indicating the presence of import mechanisms (Fusseder et al., 1989). Roots are considered to serve as the principal sites of cytokinin production, whereas the shoot depends on importing these hormones by way of the transpiration stream (Letham and Palni, 1983; Horgan, 1992). In addition, reflux of cytokinins from shoot to root through the phloem has been observed (Weiler and Ziegler, 1981).

Compared with the biosynthesis of nucleic acid bases and their derivatives, little is known about the molecular basis of transport mechanisms in eukaryotes. Only in bacteria and fungi have carrier genes for nucleic acid bases been identified. The *Escherichia coli* PurP is responsible for energized high-affinity adenine uptake (Burton, 1994). Bacterial transporters are related to the *Emericella nidulans* UapA purine permease and the UapC uric acid–xanthine permease (Gorfinkiel et al., 1993; Diallinas et al., 1995, 1998). Homologs of this family also have been identified in mammals, but their function has not been demonstrated (Faaland et al., 1998). Also related to this family is a plant membrane protein, leaf permease1 (LPE1), which seems to be involved in chloroplast function (Schultes et al., 1996). Again, a function in nucleic acid base transport has not been demonstrated. Thus far, the best-studied systems for nucleic acid base transport are the yeast ScFCY2 purine–cytosine permease, which mediates proton-coupled uptake of adenine, hypoxanthine, guanine, and cytosine (Weber et al., 1990; Bloch et al., 1992; Brethes et al., 1992; Pinson et al., 1996), and the yeast uracil permease FUR4 (Jund et al., 1988; Galan et al., 1996; Marchal et al., 1998).

As a first step toward identifying transporters for nucleic acid bases and their derivatives, such as cytokinins or secondary metabolites, the purine–cytosine transport–deficient yeast mutant *fcy2* was used to clone plant transporter genes by functional complementation. This approach led to identification and characterization of a new class of polytopic membrane proteins that mediate transport of nucleic acid bases and their derivatives.

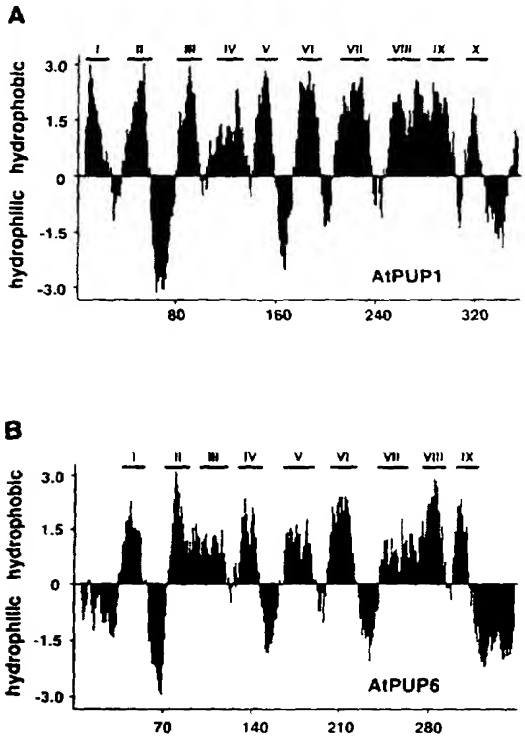

Figure 2. Prediction of Putative Membrane-Spanning Domains of AtPUP1 and AtPUP6.

Hydropathy plots were performed with a window of 11 amino acids (Kyte and Doolittle, 1982). Predicted membrane-spanning domains were confirmed by using THMM1.0 (Sonnhammer et al., 1998) and are marked as bars designated I to X **(A)** and I to IX **(B)**.
(A) AtPUP1.
(B) AtPUP6.

Table 1. Structural Features of PUP Transporter Genes and Proteins

Gene	Chromosome	Intron (Contained)	Length[a]/No. of Hydrophobic Domains	% Identity to AtPUP1	Molecular Mass[b] (kD)
AtPUP1	ND[c]	ND	357/10	100	39.2
AtPUP2	2	yes	358/10	64.0	38.1
AtPUP3	4	no	373/10	29.5	41.2
AtPUP4	4	no	419/10	30.6	46.3
AtPUP5	4	no	387/10	27.5	42.2
AtPUP6	4	yes	349/9	30.4	38.6
AtPUP7	4	yes	345/9	33.0	37.8
AtPUP8	1	no	383/10	16.9	43.2
AtPUP9	1	no	382/10	32.3	42.3
AtPUP10	5	no	358/10	26.4	39.6
AtPUP11	2	no	361/10	30.9	39.1
AtPUP12	1	no	384/10	16.6	43.7
AtPUP13	4	no	373/10	28.2	41.5
AtPUP14	1	yes	393/10	19.4	43.3
AtPUP15	1	yes	389/10	19.9	43.3

[a] Lengths are given in amino acids.
[b] Calculated molecular mass.
[c] ND, not determined.

RESULTS

Cloning of Putative Adenine/Cytokinin Transporters

The yeast *fcy2* mutant cannot grow on media containing adenine or cytosine as the sole nitrogen source because of the lack of a common uptake system (Polak and Grenson, 1973). To create uracil auxotrophy, we introduced a deletion into the *URA3* gene in the *fcy2* mutant MG887 by gene replacement. Heterologous complementation of the resulting strain MG887-1 with a cDNA library from Arabidopsis and subsequent selection on adenine-containing medium led to the identification of two independent cDNA clones mediating growth on medium containing adenine as the sole nitrogen source (Figure 1). Retransformation of the mutant with the isolated plasmids demonstrated that no mutation or reversion at a second site was responsible for suppression. DNA sequence analysis showed that both cDNAs encode the same gene product.

The longer clone (1251 bp), designated *AtPUP1* (for *Arabidopsis thaliana* purine permease1), was sequenced completely. *AtPUP1* contains an open reading frame (ORF) of 1068 bp encoding a protein of 356 amino acids with a calculated molecular mass of 39 kD. Hydrophobicity analyses (Figure 2A), performed with THMM1.0 (Sonnhammer et al., 1998), predicted 10 putative membrane-spanning domains, demonstrating that AtPUP1 is a member of a new class of small, highly hydrophobic membrane proteins. In database searches, no similarities were found to other known transporters, including nucleic acid base transporters from other organisms. However, 14 putative ORFs derived from the Arabidopsis genome project, which have 16.9 to 64.0% amino acid identity to AtPUP1, were identified (GenBank accession numbers U78721, AL021713, AC004135, AB010072, AC000132, AC005967, AC006434, AC007635, and AC007519). Even for the most distant members of the family, alignments show clearly related regions (data not shown).

The *AtPUP* genes are distributed over at least four different chromosomes (Table 1). The bacterial artificial chromosome clone AL021713 on chromosome 4 contains a repeat of five closely related *PUP* genes, indicating recent gene amplifications. In addition, a pseudogene sharing 90% identity to AtPUP5 is located between the ORFs encoding AtPUP5 and AtPUP6. The predicted PUP proteins have hydrophobicity patterns highly similar to that of AtPUP1; however, AtPUP6 and AtPUP7 lack the first putative N-terminal membrane-spanning domain, potentially indicating that they are targeted to different subcellular compartments (Figure 2B). Only AtPUP genes 2, 6, 7, 14, and 15 contain a single intron within the coding sequence, whereas the paralogs lack introns (Table 1). A phylogenetic tree based on a comparison of the 15 PUP-like sequences is depicted in Figure 3. Related sequences were found in rhododendron (GenBank accession number AF022896), tomato (GenBank accession numbers AI488700 and AI780992), cotton (GenBank accession number AI729914), and rice (GenBank accession numbers C99477, AU30775, and D46617).

Biochemical Properties of AtPUP1

Radiotracer uptake studies performed in MG887-1 expressing *AtPUP1* showed that AtPUP1 has a K_m of 30 ± 5 μM for adenine. Adenine uptake is energy dependent (addition of

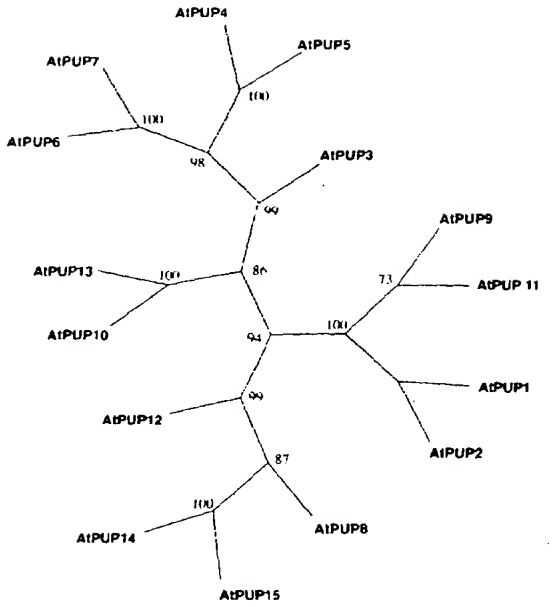

Figure 3. Tree Based on the Maximum Parsimony Analysis of the PUP Protein Sequences (Swofford, 1998).

The complete alignment was based on 465 sites, with 351 being phylogenetically informative. Percentage bootstrap values of 1000 replicates are given at each branch point.

1% glucose to the medium led to a sevenfold increase of uptake rate; data not shown), sensitive to protonophores and to H^+-ATPase inhibitors (Table 2), and increases with decreasing pH (Table 3), potentially indicating proton-coupled transport. In agreement with this hypothesis, uptake occurs against a 15-fold concentration gradient (data not shown). To study the substrate specificity of AtPUP1 in comparison with ScFCY2, we determined adenine uptake at a 10-fold molar excess of various nucleic acid bases and derivatives (Figure 4). Cytosine and hypoxanthine, but not thymine and uracil, are efficient competitors for adenine uptake. Guanine was not tested because of its low solubility.

Plants produce a wide spectrum of secondary metabolites, for example, alkaloids, some of which (e.g., nicotine, theobromine, and caffeine) share structural similarities with purines. Cytokinins also share structural similarities with purines. Both nicotine and caffeine are mobile within the vascular tissue of the plant (Dawson, 1942; Mazzafera and Gonçalves, 1999). In competition assays, the order of inhibition was adenine/kinetin/caffeine ≥ cytosine/zeatin/hypoxanthine > cytidine/nicotine > kinetin riboside/adenosine/zeatin riboside > thymine. In comparison, the nucleotides ATP, CTP, GTP, and TTP did not compete significantly for adenine uptake. Inhibition of adenine uptake by kinetin and zeatin is competitive (K_i = 20 ± 5 and 35 ± 5 μM, respectively; Figures 5A and 5B), indicating that these compounds may serve as transported substrates. Cytosine also acts as a competitive inhibitor (K_i = 30 ± 5 μM). AtPUP1 suppresses the cytosine uptake deficiency in the yeast mutant, proving that cytosine is an actual substrate (K_m = 20 ± 5 μM). Nicotine, the least-related analog tested, inhibits adenine transport by ScFCY2 and AtPUP1. However, it cannot be excluded that inhibition is due to toxicity. In comparison, yeast ScFCY2 shares many similarities regarding broad specificity toward purines and pyrimidines and selectivity toward nucleosides, but plant-specific compounds, such as caffeine or cytokinins, are not recognized by the yeast protein (Figure 4).

RNA and DNA Gel Blot Analyses of the Expression of Nucleic Acid Base Transporters

DNA gel blot analysis was conducted to demonstrate that *AtPUP1* is actually a plant gene. At high stringency, only a single locus was detected in Arabidopsis (Figure 6A), whereas under reduced stringency, additional loci were detected, which is consistent with the presence of a quite divergent gene family (Figure 6B). The expression of *AtPUP1* was analyzed under high-stringency conditions in different organs (Figure 6C). Expression of *AtPUP1* was greatest in

Table 2. Influence of Inhibitors and Protonophores on the Adenine/Cytosine Uptake Rate in MG887-1pAtPUP1[a]

Inhibitors	Adenine Uptake Rate (nmol mg DW^{-1} min^{-1})[b]	Cytosine Uptake Rate (nmol mg DW^{-1} min^{-1})
Without inhibitor	1.33	0.68
Diethylstilbestrol (100 μM)	0.046	0.027
N, N′-Dicyclohexylcarbodiimide (100 μM)	0.75	0.36
Carbonyl cyanide m-chlorphenyl-hydrazone (100 μM)	0.19	0.12
2,4-Dinitrophenol (100 μM)	0.55	0.37

[a] Adenine and cytosine concentrations were 100 μM.
[b] DW, dry weight.

Table 3. pH Dependence of Adenine (100 μM) and Cytosine (25 μM) Uptake Rate in MG887-1pAtPUP1

pH	Adenine Uptake Rate (nmol mg DW $^{-1}$ min $^{-1}$)[a]	Cytosine Uptake Rate (nmol mg DW $^{-1}$ min $^{-1}$)
4	1.14	0.35
5	0.76	0.34
6	0.48	0.14
7	0.24	0.07
8	0	0

[a] DW, dry weight.

leaves, stems, and flowers. Lower expression was found in developing siliques, whereas no expression was detectable in roots.

DISCUSSION

Suppression Cloning of Transporters

The major approach by which plant transporters have been cloned uses the suppression of uptake deficiencies in yeast mutants by functional expression of cDNA libraries (Frommer and Ninnemann, 1995). Detailed knowledge of the transport system itself is not a prerequisite. In fact, these systems are so sensitive they can even identify secondary activities of transporters that are irrelevant under physiological conditions. The extraordinary sensitivity of this suppression system is best shown in the case of NTR1. Originally identified as a histidine transporter because it complemented a histidine uptake–deficient yeast strain (Frommer et al., 1994), NTR1 was shown by a more detailed analysis to serve as a high-affinity oligopeptide transporter, with histidine transport representing only a physiologically irrelevant side activity (Rentsch et al., 1995). Because adenine is the basic structure for many compounds in the plant, attempts were made to complement a yeast adenine transport–deficient mutant. Such an approach should thus be ideal to clone transporters not only for adenine but also for the wide variety of purine analogs produced and transported in plants. This rationale is further supported by the finding that nucleic acid base transporters, such as the yeast protein ScFCY2, have low selectivity and accept purines, pyrimidines, and even nucleotides as substrates (Grenson, 1969; Pickering and Woods, 1972). Because carrier-mediated transport of cytokinins or alkaloids must be a new capacity developed during plant evolution, one might suspect that cytokinin transport systems have evolved from nucleic acid base transporters. Thus, suppression of an adenine uptake deficiency was first used to identify adenine transporters with the hope of also finding transporters for adenine analogs.

Identification of a Functional Plant Nucleic Acid Base Transporter

By using the yeast *fcy2* mutant as a sensitive complementation system, a new family (PUP) of relatively small and highly hydrophobic membrane proteins with nine to 10 putative membrane-spanning domains able to transport adenine was identified. This family consists of at least 15 quite divergent members in Arabidopsis, with related genes being present in various other plant species (e.g., rice, tomato, and rhododendron). No PUP-related sequences were found in any other kingdom of organisms until now. Given their complementation of an uptake deficiency, one may postulate that PUP proteins are located at the plant plasma membrane. The PUP family does not share significant sequence homologies with the nucleoside permease/YSPL (animal nucleic acid base transporter)/LPE nucleic acid base transporter family found in all kingdoms (Faaland et al., 1998). The putative maize nucleic acid base transporter LPE1 might serve as an organellar permease because the effects of LPE deficiency are consistent with a plastidic localization (Schultes et al., 1996). For most transporters found in plants, homologs have been identified in animals; thus, further progress in the human genome project may lead to the identification of candidates for mammalian nucleic acid base transporters related to the PUP proteins that have been postulated based on transport studies.

The adenine transporter AtPUP1 might function as a plasma membrane proton cotransporter because uptake is

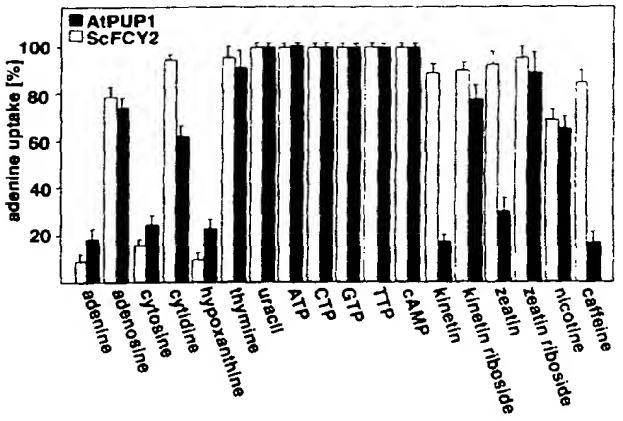

Figure 4. Substrate Specificity of AtPUP1 (in MG887-1) and ScFCY2 (Strain 1278b).

Specificity was determined by competition for uptake of ^{14}C-adenine (25 μM) with a 10-fold molar excess of nucleic acid bases, nucleosides, nucleotides, and derivatives. Data represent the mean of three independent experiments ±SD. Comparable results were obtained when ^{14}C-cytosine was used as the substrate.

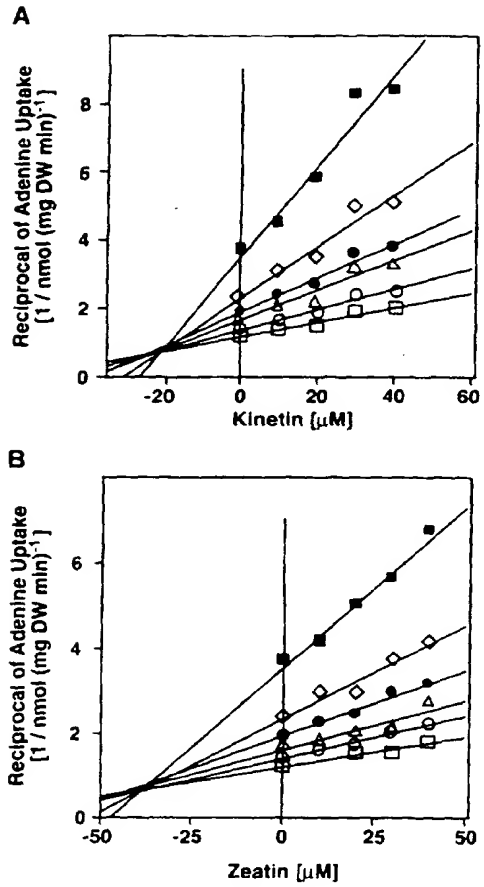

Figure 5. Competitive Inhibition of Adenine Transport by Kinetin and Zeatin.

Uptake of ^{14}C-adenine (nmol adenine per mg dry weight [DW] per minute) was determined in the presence of the competitors kinetin **(A)** and zeatin **(B)** at different concentrations of adenine (5 μM, filled squares; 10 μM, diamonds; 15 μM, filled circles; 20 μM, triangles; 30 μM, circles; and 40 μM, squares). Results are shown as Dixon plots (reciprocal of uptake rate versus inhibitor concentration). Competitive inhibition mode was derived from Lineweaver-Burke plots (data not shown).

energy dependent, occurs against a concentration gradient, and is stimulated by acidification and inhibited by protonophores. High-affinity uptake (K_m = 30 ± 5 μM) of adenine was shown directly for yeast expressing *AtPUP1*. Moreover, adenine analogs such as hypoxanthine and the pyrimidine cytosine are efficient competitors.

The expression pattern and transport properties of AtPUP1 suggest what its possible roles within the plant might be. *AtPUP1* is expressed in all tissues of the plant, except for roots, and functions in yeast as an uptake rather than an efflux system. Because cytokinin, caffeine, and nicotine are produced in roots and translocated to shoots, AtPUP1 may play a role in the uptake of these compounds from xylem sap into shoot tissues. Further studies are necessary to determine the transport properties and physiological functions of the other members of the gene family. Because adenine and zeatin can act cooperatively in flower initiation, AtPUP1 may also play a role here (Nitsch, 1968). Furthermore, nodules of tropical legumes generally export symbiotically fixed nitrogen in the form of ureides, which are produced by oxidation of de novo–synthesized purines. The greater concentration of xanthine dehydrogenase in the

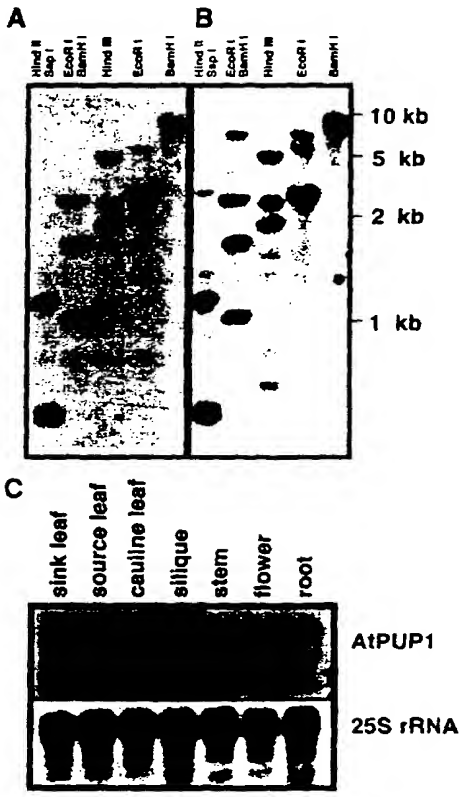

Figure 6. DNA and RNA Gel Blot Analyses of *AtPUP1*.

(A) and **(B)** High-stringency DNA gel blot **(A)** and low-stringency DNA gel blot **(B)**, obtained by using Arabidopsis genomic DNA digested with different restriction enzymes and hybridized with the radiolabeled *AtPUP1* cDNA. The positions of length markers are given at right in kilobases.

(C) RNA gel blot. RNA (30 μg) from different organs was isolated and hybridized with the radiolabeled *AtPUP1* cDNA. Control hybridization with a 25S rRNA probe is shown below.

uninfected cells suggests that xanthine or a precursor to xanthine, rather than uric acid, is the intermediate that moves from infected to uninfected cells during ureide biogenesis (Datta et al., 1991). Thus, PUP proteins may also play a role in the intercellular transport of xanthine in nodules. A detailed analysis of all members of the PUP family regarding substrate specificity and expression is required to determine the potential in vivo function of the different paralogs.

Transport of Cytokinins and Caffeine by PUP Proteins

Besides nucleic acid bases and nucleotides, derivatives such as cytokinin and alkaloids such as caffeine or nicotine are translocated in higher plants. Nucleic acid base transport plays an important role in pollen tube growth and seed germination. Cytokinin transport affects cell division, flower induction, seed germination, de novo bud formation, and senescence.

Cytokinins inhibit adenine uptake by AtPUP1 competitively with a competitive inhibition constant K_i for kinetin of 20 ± 5 μM and for zeatin of 35 ± 5 μM. Comparable values for competitive inhibition were obtained when radiolabeled cytosine was used as a substrate. Cytosine was transported with a K_m similar to the K_i. These data support the hypothesis that AtPUP1 transports cytokinins. However, they do not exclude the possibility of competitive inhibition from nontransported competitive inhibitors. Further experiments using radiolabeled cytokinins or electrophysiological measurements in Xenopus oocytes are required to prove unambiguously that AtPUP1 transports cytokinins.

However, the affinity of AtPUP1 for cytokinins as compared with concentrations in the nanomolar range for free bases, and five to 10 times greater concentrations of ribosides found in the xylem sap, may be taken as an argument against a physiological role of AtPUP1 in cytokinin transport *in vivo*. In yeast, a closely related member of the uracil permease family is responsible for uridine transport (Wagner and Beck, 1993). Conceivably, therefore, members of the PUP family are involved not only in nucleic acid base transport but also in nucleoside transport.

An argument for an actual function of AtPUP1 in cytokinin transport comes from the finding that the enzymes thought to be responsible for zeatin biosynthesis also have comparatively low affinities for their substrates. Adenine phosphoribosyltransferase, which converts adenine into AMP, also accepts zeatin as a substrate with an affinity of ~3 μM (AtTAP2) (Schnorr et al., 1996). Adenine phosphoribosyltransferase mutants are male sterile and are impaired in their metabolism of cytokinins (Gaillard et al., 1998). Similarly, adenosine kinase phosphorylates adenosine as well as isopentenyladenosine. Feeding *Physcomitrella* spp with tritiated isopentenyladenosine also resulted in the conversion of cytokinins toward their nucleotides (von Schwartzenberg et al., 1998). Together, these results demonstrate that at least two different enzymes that accept adenine or adenosine are also involved in cytokinin metabolism in vivo, despite their seemingly low affinities relative to the actual concentrations found in the plant. Thus, the results described here do not exclude the possibility that PUP proteins play a role in cytokinin transport in vivo. The actual physiological function of AtPUP1 in cytokinin transport remains to be shown, for example, by antisense repression in transgenic Arabidopsis plants.

The purine analog caffeine, which is translocated in the xylem, has also been implicated in hormonal functions. Analysis of the substrate specificity of AtPUP1 also demonstrated that caffeine competes for adenine uptake. Interestingly, transport of caffeine across the blood–brain barrier is also mediated by a side activity of the yet to be identified mammalian adenine transport protein (McCall et al., 1982).

In summary, complementation of a yeast adenine transport mutant allowed the identification of a new superfamily of transport proteins that play potential roles in the physiologically well-characterized transport of adenine. Such transport activities are potentially important in supplying pollen, germinating seeds, or the sieve elements with adenine for maintaining high ATP concentrations. A function in the transport of nucleic acid bases and hormones is also possible. Alternatively, members of the PUP protein family may be involved in transporting other secondary metabolites, such as caffeine and nicotine, the latter being synthesized in roots and translocated to leaves.

METHODS

Yeast Strains

To introduce uracil auxotrophy into the *fcy2* mutant MG887 (Mat a fcy2) (Grenson, 1969), we excised the truncated *URA3* gene (Δura3) with EcoRI and SmaI from pΔura3 (see below), and we used the Δura3 fragment for transformation of MG887. A Δura3 mutant was selected on 5-fluoro-orotic acid and named MG887-1 (Mat a fcy2 ura3). The yeast strain MG887-1 was transformed with an expression library derived from *Arabidopsis thaliana* seedlings (Dohmen et al., 1991; library in the episomal plasmid pFL61 containing the URA3 gene [Minet et al., 1992]). Transformants were selected on yeast nitrogen base without ammonium and amino acids, supplemented with 1 mg/mL adenine (7.4 mM) or cytosine (9 mM) as sole nitrogen sources. Colonies able to grow under selective conditions were reselected in liquid medium, plasmid DNA was isolated, and plasmids were reintroduced into MG887-1. The cDNAs described were able to restore growth of the mutant on adenine-containing media. Markedly better growth after complementation was obtained when *AtPUP1* was expressed under control of the *PMA1* promoter in pDR195 (Rentsch et al., 1995).

Plant Material and DNA Work

Arabidopsis ecotype C24 was grown in soil in the greenhouse. Yep24 (New England Biolabs, Beverly, MA) was digested with NcoI

and ApaI to delete an internal 173-bp fragment of the *URA3* gene, treated with T4 polymerase, and blunt-ligated to yield pΔura3 (W.N. Fischer and W.B. Frommer, unpublished results). Both strands of *AtPUP1* were sequenced with T7 polymerase (Pharmacia, Freiburg, Germany). The sequence was deposited in GenBank under accession number AF078531. DNA and RNA gel blot analyses were performed as described previously (Rentsch et al., 1996). The 25S rRNA gene served as a control (Lauter et al., 1996).

Transport Measurements

For standard uptake studies, yeast cells were harvested at OD_{600} = 0.6, washed, and resuspended in 100 mM potassium phosphate, pH 4.5, to a final OD_{600} = 12. To start the reaction, we added 100 μL of cell suspension to 100 μL of buffer containing 18.5 kBq ^{14}C-labeled adenine or cytosine (Amersham) and unlabeled analogs as indicated. Samples of 50 μL were removed after 20, 60, 120, and 180 sec, transferred to 4 mL of ice-cold water, filtered on glass fiber filters, and washed with 8 mL of water. Uptake was linear for the time period chosen (initial uptake rates were derived from these measurements). Glucose dependence was determined in the presence or absence of 200 μM glucose. The pH dependence was determined in the presence of 1% glucose in phosphate-buffered solutions. Inhibitor sensitivity was determined under comparable conditions at substrate concentrations of 100 μM. To determine the capacity of AtPUP1 to accumulate adenine or cytosine against a concentration gradient, we measured uptake over a period of 80 min. Aliquots were removed every 10 min, and cells were centrifuged, resuspended in 200 μL of buffer, filtered on glass fiber filters, and washed. Radioactivity was determined in sediment and supernatant by liquid scintillation spectrometry (Beckman). Transport measurements were repeated independently; the reported results represent the mean of at least three experiments. The almost isogenic parental strain 1278b (Mat α) served as a control (Grenson, 1969).

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ID AT9A21 standard; DNA; PLN; 82697 BP.
XX
AC AL021713;
XX
SV AL021713.1
XX
DT 03-FEB-1998 (Rel. 54, Created)
DT 23-SEP-1999 (Rel. 61, Last updated, Version 6)
XX
DE Arabidopsis thaliana DNA chromosome 4, BAC clone T9A21 (ESSA project)
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OS Arabidopsis thaliana (thale cress)
OC Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
OC euphyllophytes; Spermatophyta; Magnoliophyta; eudicotyledons;
OC core eudicots; Rosidae; eurosids II; Brassicales; Brassicaceae;
OC Arabidopsis.
XX
RN [1]
RA Bevan M., Murphy G., Ridley P., Hudson S., Bancroft I., Mewes H.W.,
RA Mayer K.F.X., Lemcke K., Schueller C.;
RT ;
RL Unpublished.
XX
RN [2]
RP 1-82697
RA EU Arabidopsis sequencing project;
RT ;
RL Submitted (22-SEP-1999) to the EMBL/GenBank/DDBJ databases.
RL MIPS, at the Max-Planck-Institut fuer Biochemie, Am Klopferspitz 18a,
RL D-82152 Martinsried, FRG, E-mail:
RL schuelle@mips.biochem.mpg.de,mayer@mips.biochem.mpg.de Project Coordinator:
RL Mike Bevan, Molecular Genetics Department, Cambridge Laboratory, John Innes
RL Centre, Colney Lane, NR4 7UJ Norwich, UK, E-mail: michael.bevan@bbsrc.ac.uk
XX
DR Demeter; AL021713; AL021713.
DR MENDEL; 27690; Arath;1535;27690.
DR MENDEL; 27693; Arath;343;27693.
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DR MENDEL; 27695; Arath;3391;27695.
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DR SPTREMBL; 049736; 049736.
DR SPTREMBL; 049737; 049737.

XP-002141276

P.D. 1998
P. Caeplet (2)

DR SWISS-PROT; 049730; MRAY_ARATH.

XP-002141277

P.D. 1996
P. Campbell (6)

ID ATU78721 standard; DNA; P 04596 BP.
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AC U78721; AE002093;
XX
SV U78721.2
XX
DT 13-DEC-1996 (Rel. 50, Created)
DT 14-JAN-2000 (Rel. 62, Last updated, Version 7)
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DE Arabidopsis thaliana chromosome II section 187 of 255 of the
DE complete sequence.
XX
KW HTG.
XX
OS Arabidopsis thaliana (thale cress)
OC Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
OC euphyllophytes; Spermatophyta; Magnoliophyta; eudicotyledons;
OC core eudicots; Rosidae; eurosids II; Brassicales; Brassicaceae;
OC Arabidopsis.
XX
RN [1]
RP 1-104596
RA Lin X., Kaul S., Rounsley S.D., Shea T.P., Benito M.-I., Town C.D.,
RA Fujii C.Y., Mason T.M., Bowman C.L., Barnstead M.E., Feldblyum T.V.,
RA Buell C.R., Ketchum K.A., Lee J.J., Ronning C.M., Koo H., Moffat K.S.,
RA Cronin L.A., Shen M., VanAken S.E., Umayam L., Tallon L.J., Gill J.E.,
RA Adams M.D., Carrera A.J., Creasy T.H., Goodman H.M., Somerville C.R.,
RA Copenhaver G.P., Preuss D., Nierman W.C., White O., Eisen J.A.,
RA Salzberg S.L., Fraser C.M., Venter J.C.;
RT "Sequence and analysis of chromosome II of Arabidopsis thaliana";
RL Nature 402:761-768(1999).
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RN [2]
RP 1-104596
RA Lin X.;
RT ;
RL Submitted (13-DEC-1999) to the EMBL/GenBank/DDBJ databases.
RL The Institute for Genomic Research, 9712 Medical Center Dr., Rockville, MD
RL 20850, USA
XX
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CC On Dec 17, 1999 this sequence version replaced gi:170700
6.
CC The sequence and annotation of chromosome 2 were merged
from those
CC of the individual clones on this chromosome after removi
ng
CC overlaps. For detailed information, please see the TIGR
web site
CC (http://www.tigr.org/tdb/at/at.html).
XX
CC Genes were identified by a combination of three methods:
Gene
CC prediction programs including GRAIL
CC (ftp://arthur.epm.ornl.gov/pub/xgrail), Genefinder (Phil
Green,
CC University of Washington), Genscan (Chris Burge,
CC http://gnomic.stanford.edu/GENSCANW.html), and NetPlantG
ene
CC (http://www.cbs.dtu.dk/services/NetGene2/), searches of
the
CC complete sequence against a peptide database and plant E
ST
CC databases at TIGR, and manual curations based on those a
nalyses.
CC Annotated genes are named to indicate the level of evide
nce for
CC their annotation. Genes with similarity to other protein
s are named
CC after the database hits. Genes without significant pepti
de
CC similarity but with EST similarity are named as 'unknown
' proteins.
CC Genes without protein or EST similarity, that are predic

ted by two

CC or more gene prediction programs over most of their leng

th are

CC annotated as 'hypothetical' proteins. Genes encoding tRN

As are

CC predicted by tRNAscan-SE (Sean Eddy,
CC http://genome.wustl.edu/eddy/tRNAscan-SE/). Simple repea

ts were

CC identified by repeatmasker (Arian Smit,
CC http://ftp.genome.washington.edu/RM/RepeatMasker.html).

Genes are

CC numbered from the top to the bottom of the chromosome.
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DT   01-NOV-1999 (TrEMBLrel. 12, Last annotation update)
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RA   Rounsley S.D., Lin X., Ketchum K.A., Phillips C.A., Brandon R.C.,
RA   Fuhrmann J.L., White O., Kerlavage A.R., Adams M.D., Somerville C.R.,
RA   Venter J.C.;
RL   Submitted (DEC-1996) to the EMBL/GenBank/DDBJ databases.
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AC H76984;

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SV H76984.1

XP-002141278

P.D. 1995

P. Caruplet (2)

XX

DT 10-NOV-1995 (Rel. 45, Created)

DT 04-MAR-2000 (Rel. 63, Last updated, Version 20)

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DE 17415 Lambda-PRL2 Arabidopsis thaliana cDNA clone 200N2T7, mRNA
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KW EST.

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OS Arabidopsis thaliana (thale cress)

OC Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
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OC Arabidopsis.

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RN [1]

RP 1-589

RX MEDLINE; 95148729.

RA Newman T., deBruijn F.J., Green P., Keegstra K., Kende H., McIntosh L.,
RA Ohlrogge J., Raikhel N., Somerville S., Thomashow M., Retzel E.,
RA Somerville C.;

RT "Genes galore: a summary of methods for accessing results from large-scale
RT partial sequencing of anonymous Arabidopsis cDNA clones";

RL Plant Physiol. 106:1241-1255(1994).

XX

DR Demeter; H76984; H76984.

XX

CC On Nov 29, 1993 this sequence version replaced gi:430448.

CC Contact: Thomas Newman
CC MSU-DOE Plant Research Laboratory
CC Michigan State University
CC MSU-DOE-PRL, Michigan State University,Plant Biology Bldg.,E.
CC Lansing,Mi
CC Tel: 517-353-0854
CC Fax: 517-353-9168
CC Email: 22313tcn@ibm.cl.msu.edu

CC Seq primer: T7 dye primer.

XX

FH Key Location/Qualifiers

FH

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FT of 4 pools of mRNA. The mRNA sources were 1) 7 day
FT germinated etiolated seedlings; 2) tissue culture grown
FT roots; 3) staged plants half with 24 hour light cycle, half
FT on 16 hr light, 8 hour dark- rosettes; 4) same plants as 3
FT but aerial tissue (stems, flowers and siliques. The vector
FT is BRL's lambda Zip-Lox. The cDNA inserts were
FT directionally cloned with Sal-Not arms using oligo dT
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Leaf permease1 Gene of Maize Is Required for Chloroplast Development

Neil P. Schultes,[a,1] Thomas P. Brutnell,[a,2] Ashley Allen,[a] Steven L. Dellaporta,[a] Timothy Nelson,[a,3] and Jychian Chen [b]

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Adjacent bundle sheath and mesophyll cells cooperate for carbon fixation in the leaves of C4 plants. Mutants with compromised plastid development should reveal the degree to which this cooperation is obligatory, because one can assay whether mesophyll cells with defective bundle sheath neighbors retain C4 characteristics or revert to C3 photosynthesis. The *leaf permease1-mutable1* (*lpe1-m1*) mutant of maize exhibits disrupted chloroplast ultrastructure, preferentially affecting bundle sheath choroplasts under lower light. Despite the disrupted ultrastructure, the metabolic cooperation of bundle sheath and mesophyll cells for C4 photosynthesis remains intact. To investigate this novel mutation, the *Activator* transposon-tagged allele and cDNAs corresponding to the *Lpe1* mRNA from wild-type plants were cloned. The *Lpe1* gene encodes a polypeptide with significant similarity to microbial pyrimidine and purine transport proteins. An analysis of revertant sectors generated by *Activator* excision suggests that the *Lpe1* gene product is cell autonomous and can be absent up to the last cell divisions in the leaf primordium without blocking bundle sheath chloroplast development.

INTRODUCTION

The photosynthetic leaf cells of higher plants rely on many intracellular and intercellular interactions for their differentiation and function. Nuclear, plastid, and mitochondrial gene expression are coordinated for the development and metabolism of photosynthetic cells (Mullet, 1988). Intracellular interactions in the form of metabolite transfer between cytosol and organelles maintain photosynthesis, respiration, and other metabolic processes in differentiated leaf cells (Raghavendra et al., 1994). In addition, intercellular interactions are particularly important in plants that perform C4 carbon fixation, a process dependent on the metabolic cooperation and structural differentiation of two dissimilar photosynthetic cells. Leaves of C4 plants, such as maize, display Kranz anatomy, in which veins are surrounded by an inner layer of photosynthetic bundle sheath cells and an outer layer of photosynthetic mesophyll cells. Each cell provides a unique subset of the required enzymatic activities for C4 photosynthesis, due mainly to cell-specific expression of the genes encoding C4 pathway enzymes (Laetsch, 1974; Nelson and Langdale, 1992, 1993). The differentiation and photosynthetic function of bundle sheath and mesophyll cells rely on light and on positional information that is as yet poorly understood. In the absence of light and positional context, photosynthetic cells differentiate with a "default" pattern of enzymatic activities suitable for C3 carbon fixation (reviewed in Nelson and Langdale, 1992, 1993).

A genetic approach provides the means to interrupt and dissect the intracellular and intercellular interactions that direct C4 cell development and function. Maize is well suited for mutational analysis of this pathway because the large seed endosperm supports substantial leaf development in plants with severe metabolic or developmental defects. The distinct cellular morphologies and metabolic roles of bundle sheath and mesophyll cells make it feasible to recover mutations with bundle sheath– or mesophyll-specific phenotypes. Among these should be mutations in "upstream" genes that influence cell identity and in "downstream" genes that perform cell differentiation. Mutations in both classes should affect bundle sheath and mesophyll cell interactions. Although many pigment-deficient, seedling-lethal, and leaf pattern mutations have been described in maize and other C4 plants, few mutations of these two classes have been identified. A potential member of the upstream class, *bundle sheath defective1-mutable1* (*bsd1-m1*), affects bundle sheath chloroplast development but not neighboring mesophyll cell chloroplasts (Langdale and Kidner, 1994). The C4 pattern of enzyme accumulation in *bsd1-m1* plants is disrupted early in development and before light-activated plastid maturation, suggesting that the wild-type gene has a regulatory role. Cell-specific or cell-preferential defects likely to fall in the downstream class are found among the many maize high chlorophyll fluorescence

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(*hcf*) mutants. For example, the *hcf3* mutant is deficient in mesophyll cell–specific photosystem II thylakoid complexes (Metz and Miles, 1982).

Here, we characterize a mutation, *leaf permease1* (*lpe1*), that affects both bundle sheath and mesophyll chloroplasts under field conditions but preferentially affects bundle sheath chloroplasts under lower light intensity. The *lpe1-m1* allele was generated by insertional mutagenesis with the maize transposable element *Activator* (*Ac*), facilitating cloning of the disrupted gene. A cDNA corresponding to wild-type *Lpe1* mRNA encodes a hydrophobic polypeptide with significant similarity to bacterial uracil permeases and to a fungal xanthine transporter. Both the structure of the predicted protein and the phenotype of the *lpe1-m1* mutant suggest that the gene product is cell autonomous and essential for full chloroplast development. Although photosynthetic capacity is compromised, mutant bundle sheath and mesophyll cells are still linked metabolically for C4 photosynthesis.

RESULTS

lpe1-m1 Phenotype

A mutation showing a somatically unstable pale green phenotype segregated in a screen of M_2 families from individual selfed kernels enriched for *Ac* transpositions from the *P-vv* locus on chromosome 1S in maize (Dellaporta and Moreno, 1993). Plants homozygous for the *lpe1-m1* allele displayed a somatically unstable pale green phenotype. Sectors of normal dark green tissue appeared on a background of pale green mutant tissue. The dark green somatic sectors were large, presumably representing *Ac* excision events early in leaf development, as seen in Figure 1A, or were numerous and small, indicative of *Ac* excision late in leaf development, as seen in Figure 1B. Mutants grew to maturity with large revertant sectors or with numerous small revertant sectors. However, mutants with few revertant sectors only survived under greenhouse conditions, remained very small, and failed to produce functional floral organs.

The *lpe1-m1* pigment-deficient phenotype is light intensity–dependent. At low light intensity (50 $\mu E\ m^{-2}\ sec^{-1}$), the mutant seedling leaf tissue was visibly indistinguishable from that of wild-type siblings, whereas at higher light intensity (300 $\mu E\ m^{-2}\ sec^{-1}$), mutant leaf tissue was pale green and clearly discernible from wild-type tissue. Under field conditions (~2000 $\mu E\ m^{-2}\ sec^{-1}$), mutant tissue appeared pale green to yellow. The chlorophyll content of mutant leaf tissue was significantly lower than that of wild-type siblings at both 50 and 300 $\mu E\ m^{-2}\ sec^{-1}$, as shown in Table 1.

lpe1-m1 plants exhibited a cell-autonomous, defective-chloroplast phenotype most apparent in bundle sheath cells. Mutant and revertant bundle sheath cells were easily distinguished at the light microscope level of resolution due to the differences in plastid size and number. In mature, field-grown leaf blade tissue, bundle sheath cells in mutant sectors contained abnormally small chloroplasts, as shown in Figure 2, and ~50% fewer chloroplasts than in revertant sectors (data not shown). In contrast to the striking difference between mutant and revertant bundle sheath chloroplasts, revertant-sector mesophyll chloroplasts stained with only slightly greater intensity than did neighboring mutant mesophyll chloroplasts. In dark green revertant sectors, chloroplasts of bundle sheath and mesophyll cells were indistinguishable from those in wild-type sibling plants. The mutation did not appear to alter overall leaf architecture, vasculature, or Kranz anatomy.

To determine whether bundle sheath cells contained a mixture of wild-type and mutant chloroplasts, ~1000 bundle sheath cells were reconstructed from serial transverse sections from

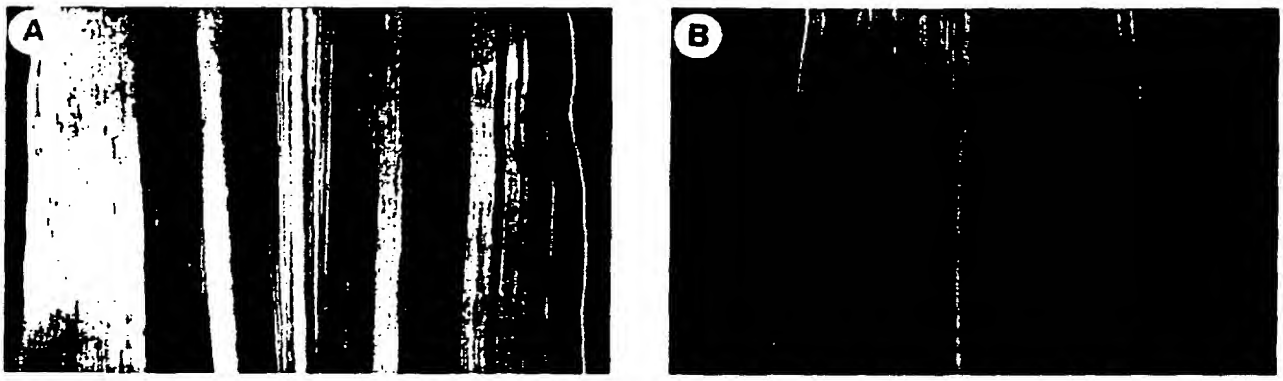

Figure 1. *lpe1-m1/lpe1-m1* Leaf Phenotype.

Field-grown adult leaves of *lpe1-m1* plants display somatic instability of dark green revertant sectors (in black) on a yellow to pale green mutant background (gray region).

(A) Revertant sectors that are large and infrequent.

(B) Revertant sectors that are small and numerous.

Table 1. Leaf Chlorophyll Concentration and Light Intensity

Light Intensity	Mutant	Wild Type	Significance[a]
50 μE m $^{-2}$ sec $^{-1}$	2.74 (± 0.415) μg chlorophyll *a* and *b* per mg leaf tissue	3.28 (± 0.484) μg chlorophyll *a* and *b* per mg leaf tissue	$P < 0.015$
300 μE m $^{-2}$ sec $^{-1}$	1.51 (± 0.183) μg chlorophyll *a* and *b* per mg leaf tissue	2.17 (± 0.0309) μg chlorophyll *a* and *b* per mg leaf tissue	$P < 4 \times 10^{-6}$

[a] Student's *t* test for the difference between the mutant and wild type at a given light intensity.

a mature, field-grown leaf blade containing mutant and revertant sectors (data not shown). Individual bundle sheath cells were found to contain plastids of either mutant or wild-type morphology but never a mixture of both. In addition, many single bundle sheath cell revertants were observed, representing reversion events occurring very late in leaf development. This finding suggests that the *Lpe1* gene product can act relatively late in development. The clear morphological distinction between revertant and mutant bundle sheath cells suggests that the *Lpe1* gene product acts in a cell-autonomous manner.

Plastids of Mutant Mesophyll and Bundle Sheath Cells Differ in Ultrastructural Defects

The chloroplasts of bundle sheath and mesophyll cells from mature, field-grown mutant leaf blade tissue were examined by transmission electron microscopy. Bundle sheath chloroplasts in mutant tissue were small (4.1 μm average length), contained few or no starch granules, and had on average 15 unstacked thylakoids, as represented in Figure 3A. These mutant sausage-shaped organelles had numerous plastoglobuli (carotenoid-containing bodies) at either pole and were enveloped by a swollen peripheral reticulum, which in many cases consisted of four or five layers. The peripheral reticulum is a layered membrane elaboration of unknown function often associated with chloroplasts of C4 plants (Douce et al., 1985) but normally in less abundance than observed in these mutant bundle sheath chloroplasts. Mesophyll chloroplasts in mutant tissue were of normal size and shape but lacked the extensive granal stacks characteristic of normal mesophyll chloroplasts, as seen in Figure 3B. In contrast, bundle sheath and mesophyll chloroplasts in revertant sectors appeared normal in morphology. Bundle sheath chloroplasts were large (8 mm average length), had ~20 unstacked thylakoids, were packed with numerous large starch granules, and did not have a swollen peripheral reticulum, as represented in Figure 3C. Mesophyll chloroplasts in revertant sectors appeared normal, with highly stacked grana (Figure 3C). The mitochondria in mutant bundle sheath and mesophyll cells appeared normal in size and morphology (data not shown).

The plastid phenotype appeared to be bundle sheath–specific at lower light intensity, although the alterations were less severe. We examined the chloroplasts of mutant seedlings grown at 1000 μE m^{-2} sec^{-1} illumination (half solar), a level sufficient to cause extensive photodamage in most carotenoid-deficient mutants. Figure 3D shows that mutant mesophyll cell chloroplasts grown under half-solar illumination contained stacked grana, similar in appearance to the wild type. Mutant bundle sheath chloroplasts were small, had few thylakoids, and lacked large starch granules but did not exhibit excessive peripheral reticulum or numerous plastoglobuli, as shown in Figure 3E. The swollen peripheral reticulum in bundle sheath chloroplasts and the unstacked grana of mesophyll chloroplasts in field-grown mutant tissue may have resulted from secondary damage and occurred only at high light intensity, possibly in association with the observed pigment deficiency. It is also possible that the differences observed in mutant chloroplasts grown under different light intensities (compare Figures 3A and 3B with Figures 3D and 3E) are due either to the different developmental ages of leaf tissue or to a combination of leaf age and light intensity.

Figure 2. Histological Examination of *lpe1-m1* Leaf Tissue.

A transverse section through mature *lpe1-m1* tissue is shown. Revertant bundle sheath cells (closed arrow) contain wild-type chloroplasts. Mutant bundle sheath cells (open arrow) contain small, poorly staining chloroplasts. Mesophyll cells in revertant sectors contain chloroplasts that stain slightly more than chloroplasts in mesophyll cells of mutant sectors.

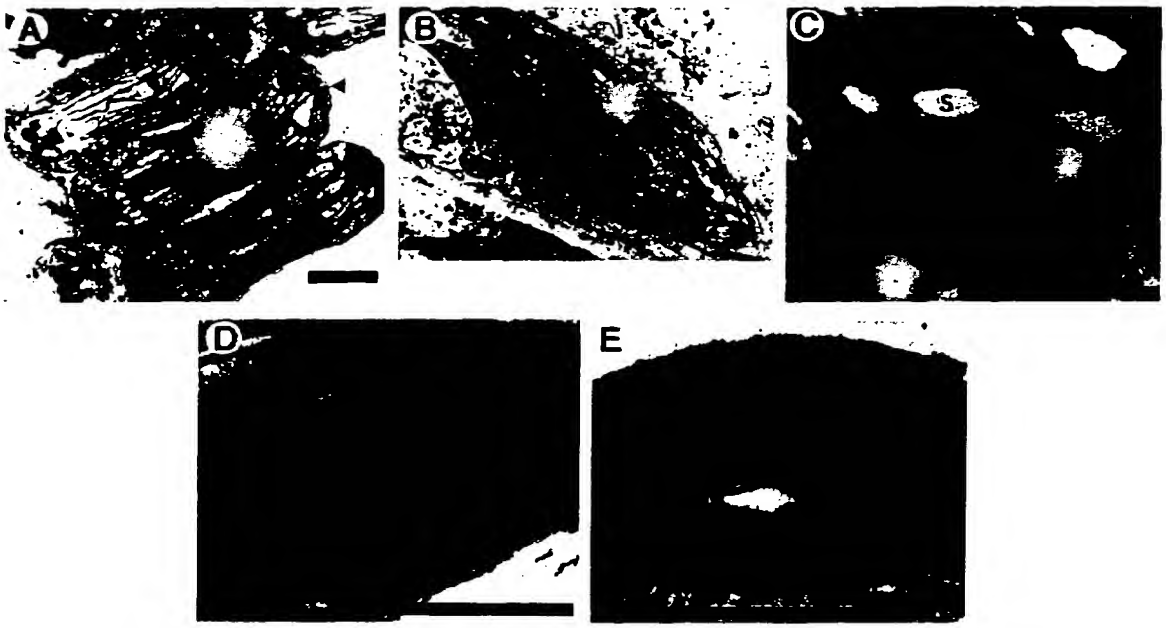

Figure 3. Chloroplast Ultrastructure.

(A) Mutant bundle sheath chloroplasts. Sections were prepared from mature, field-grown *lpe1-m1* tissue. Swollen peripheral reticulum (closed arrowhead) is present at the chloroplast border, few small starch granules are present, and plastoglobuli (open arrowhead) segregate to poles.
(B) Mutant mesophyll chloroplast. Sections were prepared from mature, field-grown *lpe1-m1* tissue. Note the lack of highly stacked grana.
(C) Revertant-bundle sheath and mesophyll chloroplasts. Sections were prepared from field-grown *lpe1-m1* revertant-sector tissue. Note the lack of swollen peripheral reticulum, the numerous thylakoids and large starch granules (S) in bundle sheath chloroplasts, and the highly stacked grana (g) in mesophyll chloroplasts.
(D) Mutant mesophyll chloroplast. The section was prepared from *lpe1-m1* mutant sector tissue of seedlings grown at 1000 μE m^{-2} sec^{-1} illumination. Note the highly stacked grana (g).
(E) Mutant bundle sheath chloroplast. The section was prepared from *lpe1-m1* mutant sector tissue of seedlings grown at 1000 μE m^{-2} sec^{-1} illumination. Note the lack of excessive peripheral reticulum yet the lack of substantial starch granules.
Bars in **(A)** to **(E)** = 1 μm.

lp 1-m1 Is Caused by *Ac* Element Insertion

Several lines of evidence indicate that an *Ac* element disrupts *Lp 1* and is responsible for the mutant phenotype. First, the mutation arose in a nondirected transposon-tagging mutagenesis in which M_1 seed were enriched for both *Ac* transposition and reinsertion elsewhere in the genome, as described by Dellaporta and Moreno (1993). Second, the somatic instability of the *lpe1-m1* mutation (Figures 1A and 1B) is characteristic of transposon-induced leaf phenotype mutations, such as *pale green14*, *bundle sheath defective1*, and *yellow1-mum* (Peterson, 1960; Buckner et al., 1990; Langdale and Kidner, 1994), which display randomly placed revertant sectors across the leaf. Third, the frequency and amount of *Lpe1* revertant sectors depended on *Ac* dosage. As the copy number of *Ac* elements in the genome increased, the frequency of revertant sectors decreased (data not shown). Fourth, *Ac* was genetically linked to the mutation. Figure 4B shows that a novel *Ac*-containing genomic fragment, lpe-m, was found in the DNA of all mutants (lanes 1 to 6), and in that of a subset of phenotypically wild-type siblings (presumed heterozygotes) (lanes 7, 9, 11, and 12), as expected for a recessive mutation. Fifth, *Ac* excision from *Lpe1* was associated with the presence of wild-type revertant sectors in the mutants. Mutant tissue should contain two *lpe1-m1* chromosomes, whereas revertant tissue will be typically heterozygous for the *lpe1-m1* and *Lpe1*-revertant chromosomes (Figure 4A).

DNA gel blot analysis of genomic PstI-digested DNA probed with an *Lpe1*-specific probe showed that mutant tissue contained predominantly the *Ac*-containing fragment (Figure 4C, lanes 1, 5, and 7), whereas the revertant tissue had both *Ac*-containing and *Ac*-excision fragments (lanes 2 to 4, 6, and 8). The difference between the mutant fragment and the revertant fragment was approximately the length of the *Ac* element (4.5 kb). The proportion of the revertant *Lpe1* fragment within phenotypically mutant tissue (e.g., Figure 4C, lanes 1 and 7) corresponded approximately to the amount of small, dark green revertant sectors observed in the mutant tissue (compare

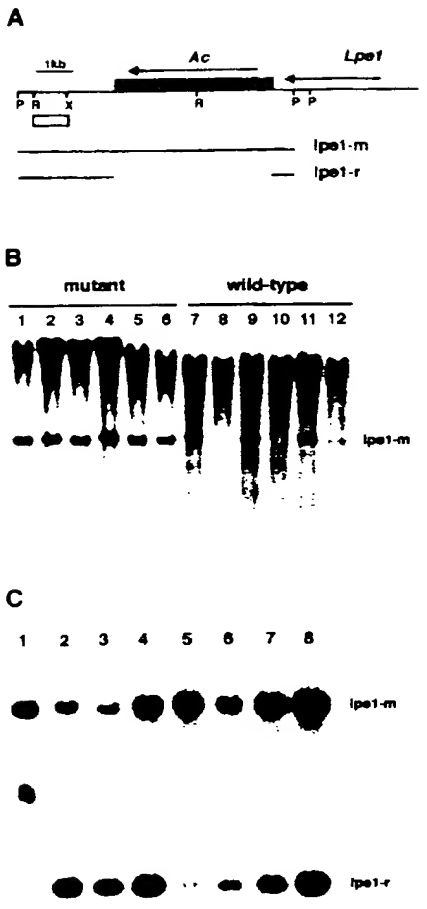

Figure 4. The *Lpe1* Gene Is Tagged by an *Ac* Element.

(A) *Lpe1-m1* DNA representation. The solid box represents the *Ac* element inserted into the *Lpe1* gene (solid line). Arrows represent the direction of transcription for the *Ac* element and *Lpe1* gene, based on sequence analysis. The open box denotes the *Lpe1*-specific 3′ genomic fragment used as a probe for DNA and RNA gel blot analyses. lpe1-m indicates the 7.9-kb PstI restriction endonuclease fragment of the *Ac*-disrupted *Lpe1* gene. lpe1-r indicates the 3.1-kb PstI restriction endonuclease fragment of the revertant *Lpe1* gene without the *Ac* insertion (see **[B]** and **[C]**). P, PstI; R, EcoRI; X, XhoI.

(B) Segregation analysis. A novel *Ac* restriction endonuclease fragment segregated among *lpe1-m1*-bearing F_2 seedlings. Lanes 1 to 6 contain genomic DNA isolated from *lpe1-m1* seedlings digested with the PstI restriction endonuclease. Lanes 7 to 12 contain PstI-digested DNA from phenotypically wild-type siblings. The blot was probed with internal *Ac* sequences (see Methods).

(C) Revertant-sector analysis. DNA from mutant (lanes 1, 5, and 7) and revertant sectors (lanes 2 to 4, 6, and 8) in three mutants (1086-1,1194-7, and122-14) was cleaved with the PstI restriction endonuclease and probed with the *Lpe1*-specific 3′ genomic fragment (shown in **[A]**). lpe1-m and lpe1-r refer to the *Ac*-containing and *Ac* excision fragments described in **(A)**.

Figures 1A and 1B). Pale green tissu was not expected to be ntirely isogenic due to the combination of late *Ac* xcision events resulting in small sectors and phenotypically invisible revertant sectors of any size in the epidermis. Sixth, sequence comparison of the *Ac*-tagged *Lpe1* gene and the corresponding cDNA revealed that the novel *Ac* element had inserted into the middle of an intron, as indicated in Figure 5A. Excision of the *Ac* element from *Lpe1* frequently restored the function of the gene, as demonstrated for an *Ac* insertion at the *P* locus (Brink and Nilan, 1952).

Lpe1 Locus Encodes a Novel Protein with Similarity to Pyrimidine and Purine Transport Proteins

Several *Lpe1*-hybridizing clones were isolated from a maize seedling green leaf cDNA library. The DNA sequence of the longest clone is shown in Figure 5A. The indicated ATG codon most likely serves as a translational start because it is preceded by three stop codons in each of three reading frames and initiates a 1464-bp open reading frame followed by a 421-bp 3′ untranslated region. The *Lpe1* gene lacks two features commonly found in highly expressed plant genes: the initiation ATG context does not conform to the consensus sequences derived from highly expressed plant genes (Joshi, 1987), and the proposed 5′ untranslated region is GC rich (68%). Consistent with this finding, we showed that the gene encodes a rare mRNA found in leaves and roots (see below).

The protein predicted by the *Lpe1* cDNA consists of 487 amino acids with a predicted molecular mass of 53,228 D. Several features suggest that the Lpe1 protein is an integral membrane protein. Figure 5B shows that the predicted protein is extremely hydrophobic, consists of 47.2% hydrophobic amino acids, and displays only a small central hydrophilic region (from approximately amino acids 216 to 266). Many membrane-associated regions were predicted in this polypeptide sequence. Protein structure algorithms predicted either 10 membrane-spanning segments (Klein et al., 1985), 12 membrane-associated helices (seven transmembrane, three surface, and two globular) (Eisenberg et al., 1984), or five transmembrane helices (Rao and Argos, 1986). As expected for integral membrane proteins (Klein et al., 1985), DNA binding motifs or coiled-coil regions were not found. Potential mitochondrial and chloroplast transit peptide motifs and cleavage sit s were not apparent in the Lpe1 protein sequence (von Heijne et al., 1989; Gavel and von Heijne, 1990a, 1990b).

The Lpe1 protein showed a limited but significant amino acid sequence similarity to several membrane-associated permease proteins. Statistically significant similarity was found to uracil permeases from *Bacillus caldolyticus*, *B. subtilis*, and *Escherichia coli*, to hypothetical proteins from *B. subtilus* and *Clostridium perfringens*, and to a uric acid–xanthine permease from the filamentous fungus *Aspergillus nidulans* (Figure 5B). The region of gr at st similarity, between amino acids 345 and 432 (Figure 5C), included a membrane-spanning segment predicted by all three aforementioned algorithms. S veral

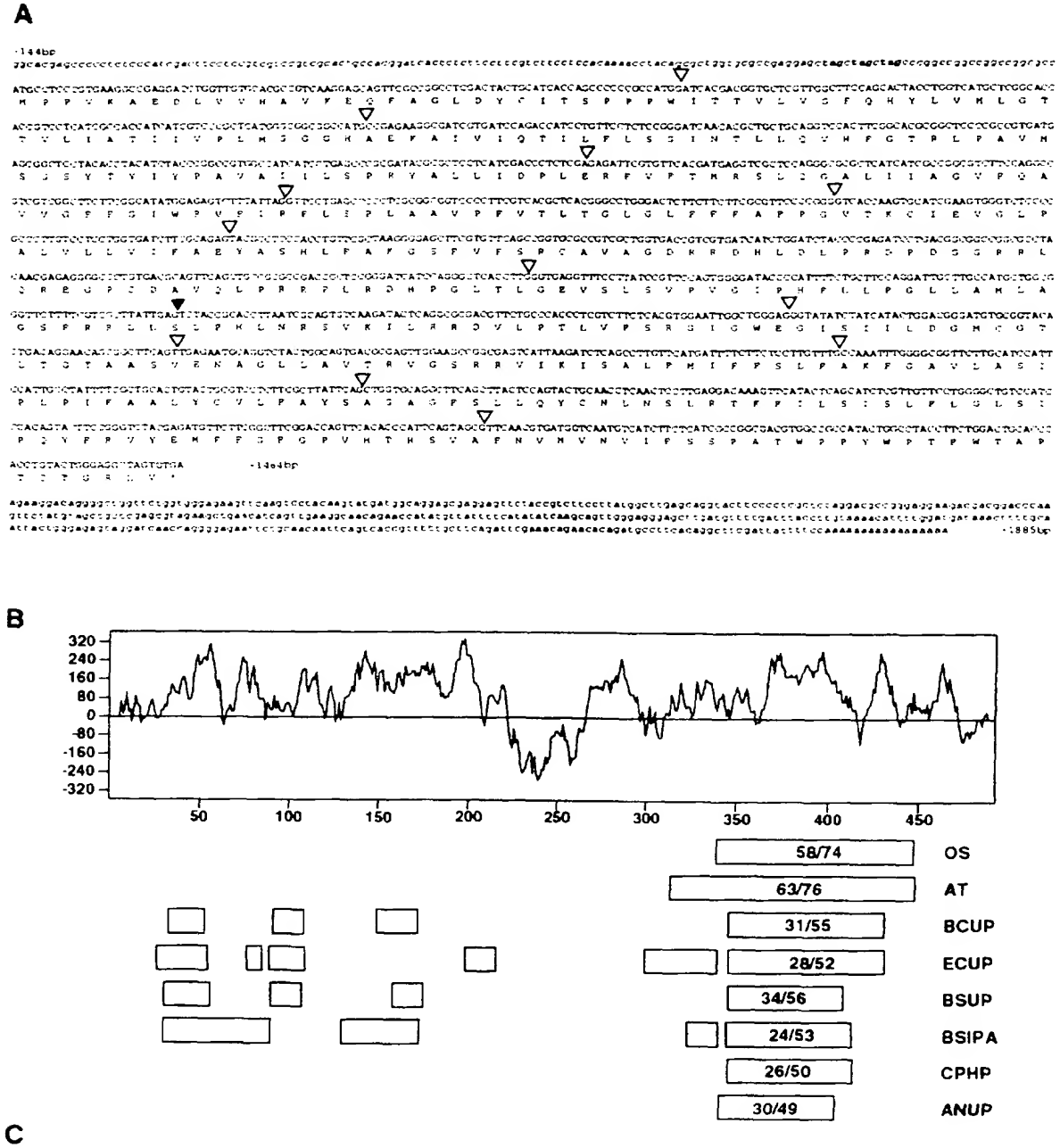

Figure 5. Sequence Analysis.

smaller patches of similarity were found in colinear fashion between the Lpe1 protein and the *Bacillus* and *Escherichia* uracil permeases (Figure 5B), suggesting a similar domain organization. The Lpe1 protein was highly similar to proteins predicted by rice and Arabidopsis-expressed sequence tag clones (Figures 5B and 5C).

Lpe1 mRNA Accumulation

Figure 6 shows that an *Lpe1* gene probe detected low levels of a 1.9-kb transcript in roots and in dark-grown leaves but not in green leaves from wild-type plants. The steady state level was approximately six- to eightfold lower in dark-grown leaves than in roots, based on digitized image analysis of autoradiographs. The *Lpe1* transcript was more abundant in the root and shoot of young seedlings germinated in the dark for 3 days than in the expanded portions of 10-day-old etiolated seedling leaves (data not shown). The *Lpe1* transcript is present in dark-grown leaves (Figure 6A, lane 2) but is less abundant after 24 hr of illumination (Figure 6A, lane 3). The transcript remains undetectable in light-grown leaves 12, 24, or 48 hr after a shift to darkness (Figure 6A, lane 5, for the 24-hr time point; 12- and 48-hr data not shown).

Mapping and Allelism Tests for *Lpe1*

The *lpe1* locus was mapped to chromosome 1L by using both recombinant inbred lines and B-A translocation lines. *lpe1-m1* heterozygotes were crossed to B-A translocation lines with break points on chromosome 1S or 1L. Of the F_1 progeny from such crosses, 6% (8/133) were pale green and mitotically unstable. The mutable pale green plants had the runty architecture and slender leaves characteristic of chromosome 1 hypoploid plants, suggesting that the recessive allele had been "uncovered" by the loss of the wild-type allele on 1L. The *Lpe1* locus was more precisely located to chromosome 1L (map position 88) by DNA gel blot analysis of recombinant inbred families (Burr et al., 1988). The *Lpe1* locus was 12.5 centimorgans (cM) from marker *bnl*7.06, 18.7 cM from the *Bronze2* locus, and 23.3 cM from marker *bnl*8.10.

Complementation tests were performed between *lpe1-m1* and several other mutations on chromosome 1. F_1 seedling analysis revealed that the *lpe1-m1* mutation was not allelic to *pale green15* (chromosome 1S), or to *pale green16*, *green stripe1*, *white luteus5*, or *lemon white1* (chromosome 1L), or to the adjacent *fine stripe1* (1L-86) to a significance of $P < 0.01$. In addition, genetic and molecular tests showed that *lpe1* is not allelic to *bsd1* (J. Langdale, personal communication). Because the *lpe1-m1* mutants did not exhibit high chlorophyll fluorescence when dark adapted and irradiated with UV illumination, no complementation tests were performed with the many *hcf* mutations that map to chromosome 1L.

Bundle Sheath and Mesophyll Cells Continue to Cooperate for C4 Metabolism in *lpe1-m1* Tissue

The apparent disruption of bundle sheath chloroplasts in *lpe1-m1* homozygotes provided an opportunity to test the metabolic cooperation of bundle sheath and mesophyll cells. We reasoned that development of photosynthetic metabolism in mesophyll cells may compensate for loss of its bundle sheath C4 partner by reverting to C3-type carbon fixation (Langdale et al., 1988). Alternatively, mutant tissue may fail to perform photosynthesis or may perform C4 photosynthesis at a greatly reduced level. These possibilities can be distinguished by

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Figure 5. (continued).

(A) *Lpe1* cDNA sequence. Nucleotides in boldface represent amber stop codons **(tag)** preceding the large open reading frame. Open triangles indicate the insertion point of known introns, and the solid triangle indicates the start of the intron in which the *Ac* element inserted in the *Lpe1* gene. This intron is 267 bp long, and the *Ac* element is located between 138 and 139 bp 5′ to 3′. Lowercase letters denote nucleotides in the proposed untranslated regions, whereas uppercase letters denote nucleotides in the proposed coding region. Amino acids given in the single-letter abbreviation below DNA codons denote the sequence of the proposed Lpe1 protein. The GenBank accession number is U43034.

(B) Lpe1 protein homology and hydropathy plot (Kyte and Doolittle, 1984). Numbers along the plot's bottom border indicate the amino acid position in the Lpe1 protein. Open bars below the plot represent areas of amino acid similarity between the Lpe1 protein and other plant, bacterial, and fungal proteins, as determined by BLAST analysis (Altshul et al., 1990). OS, *Oryza sativa* translation of an expressed sequence tag (DDBJ accession number D28174); AT, Arabidopsis, translation from a partial sequence from an expressed sequence tag (EMBL accession number Z25495); BCUP, ECUP, and BSUP, uracil permeases from *B. caldolyticus* (Ghim and Neuhard, 1994) (EMBL accession number X76083), *E. coli* (Andersen et al., 1995) (EMBL accession number X73586), and *B. subtilus* (Turner et al., 1994) (GenBank accession number M59757), respectively; BSIPA, *B. subtilus* hypothetical open reading frame ipa-60d (Fujita et al., 1986; Glaser et al., 1993) (EMBL accession number X73124); CPHP, *C. perfringens* hypothetical protein (Brynestad et al., 1994) (PIR accession number S33349); ANUP, *A. nidulans* uric acid–xanthine permease (Gorfinkiel et al., 1993) (EMBL accession number X71807). The numbers in the open boxes represent the percentage of amino acid identity/amino acid similarity. All P values for amino acid similarity are < 0.05.

(C) Amino acid homology alignment. Shown is an expanded pairwise BLAST alignment of Lpe1 protein amino acids 339 to 429 and similar proteins (Altshul et al., 1990). Protein sequences are labeled as given in **(B)**. (+) indicates amino acid similarity; an empty space indicates an amino acid sharing no identity or similarity with the Lpe1 protein and the compared sequence; the protein sequences are linear and have no gaps in the comparison. For the Arabidopsis sequence, several open reading frames were spliced together to overcome probable DNA sequence errors.

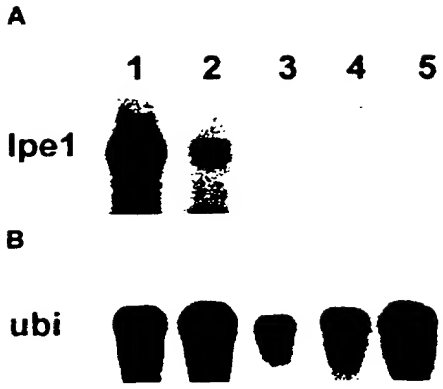

Figure 6. *Lpe1* mRNA Accumulation.

(A) *Lpe1* RNA after light shifts. RNA gel blot of poly(A)$^+$ mRNA (1 μg) isolated from seedling roots (lane 1), etiolated seedling leaves (lane 2), etiolated seedling leaves shifted to 24 hr of light (lane 3), green seedling leaves (lane 4), and green seedling leaves shifted to 24 hr of darkness (lane 5). A 1.9-kb transcript is visible in roots and etiolated leaves (lanes 1 and 2) but not in green leaves (lanes 4 and 5). The blot was probed with an *Lpe1*-specific 3′ genomic probe (see Figure 4A).
(B) Control duplicate blot probed with maize polyubiquitin (ubi) sequences.

measuring the CO_2 compensation point of the mutant tissue. In a closed system, the CO_2 compensation point is a measure between the equlibrium of CO_2 fixation by photosynthesis and release through photorespiration. C3 plants attain an equilibrium CO_2 concentration of ~50 to 80 ppm in a closed environment, whereas C4 plants achieve levels of 1 to 10 ppm due to the low rate of photorespiration. Tissue that does not photosynthesize (e.g., roots) will generate increasing levels of CO_2 due to respiration.

CO_2 compensation points were measured for leaf tissue from both seedling and adult *lpe1-m1* mutants and sibling wild-type plants. Figures 7A and 7B show that mutant tissue achieves a low equilibrium CO_2 concentration similar to that for wild-type tissue and characteristic of functional C4 carbon fixation. However, the mutant tissue achieved equilibrium at a slower rate than did the wild-type tissue (Figures 7A and 7B), suggesting that the photosynthetic rate is reduced. To determine whether revertant leaf tissue also performs C4 photosynthesis, a leaf sample from a large revertant sector on a mature greenhouse-grown *lpe1-m1* mutant plant was compared with adjacent mutant tissue in CO_2 compensation point assays. The results in Table 2 show that both the developmentally paired mutant and the revertant tissue achieve low CO_2 compensation points (< 6 ppm). Greenhouse-grown plants were used for the measurements to reduce contributions resulting from high light intensity damage. To ensure that the compensation point of mutant tissue was not influenced by contributions from significant patches of revertant tissue, DNA derived from the same revertant and mutant tissue sectors was subjected to DNA gel blot analysis, using an *Lpe1* probe. No revertant bands were detectable in the mutant tissue (data not shown), suggesting that any contribution by revertant tissue was small.

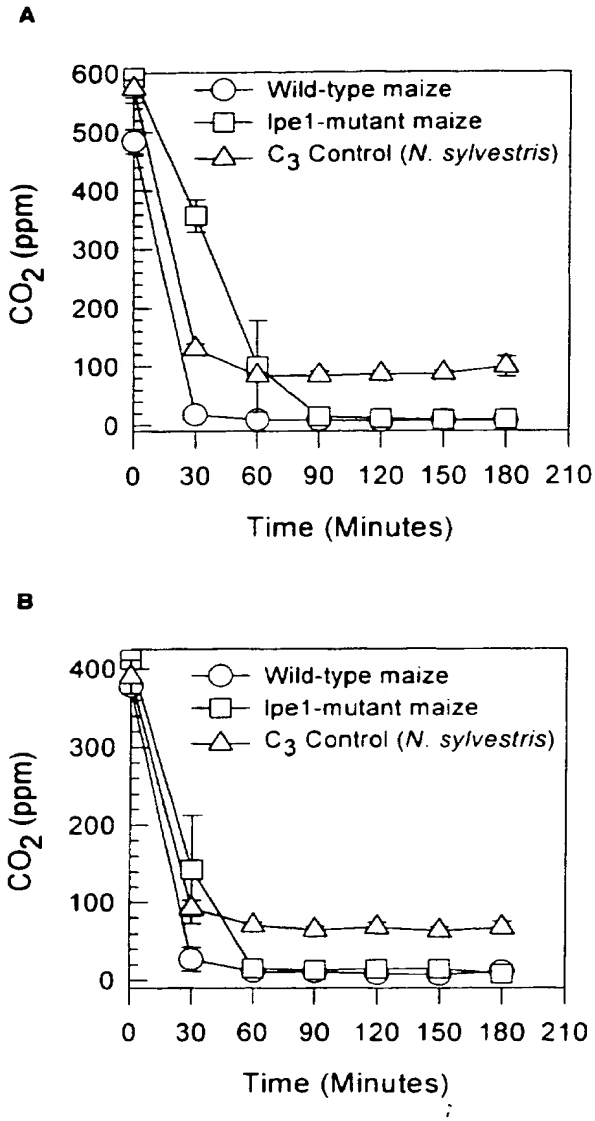

Figure 7. CO_2 Compensation Point Assays.

(A) Time course of averaged CO_2 concentration measurements for *lpe1-m1* and sibling wild-type seedling leaf tissue. *N. sylvestris* leaf tissue is a C3 control. CO_2 concentration was measured in parts per million (ppm).
(B) Time course of averaged CO_2 concentration measurements for *lpe1-m1* and sibling wild-type adult leaf tissue.

Table 2. CO_2 Compensation Point Analysis

Plant	Phenotype	CO_2 (ppm)
N. sylvestris	C3 photosynthesis control	55.2; 58
Maize	Revertant sector *Lpe1-r/lpe1-m1*	6; 3.6
Maize	Mutant sector *lpe1-m1/lpe1-m1*	1.7; 1.3

DISCUSSION

The C4 carbon fixation cycle relies on intercellular cooperation between bundle sheath and mesophyll cells and on intracellular cooperation between chloroplast and mitochondrion. Mutations that specifically or preferentially affect the function of bundle sheath or mesophyll cells represent genetic tools that can be used to investigate these interactions. We isolated one such mutation, *lpe1-m1*, which differentially affects bundle sheath and mesophyll chloroplast morphology, depending on the light intensity. The corresponding *Lpe1* gene, named for the leaf phenotype and permease homology, appears to encode an integral membrane protein that is required for normal chloroplast function.

Transposon-Induced Mutations and the Study of Gene Action

Transposable element insertions can greatly facilitate the functional analysis of target genes. First, the availability of a transposon-induced mutant allele often permits the molecular cloning of the gene by using the transposon as a molecular tag. Second, the somatic instability of many transposon-induced alleles allows the requirement for a cell-autonomous gene product to be defined in developmental time. For *lpe1-m1* plants, revertant dark green sectors reflect excision of the *Ac* element during cell divisions in the developing leaf primordium. Because most cell divisions in the leaf promordium are completed before exposure to light, the range of observed sectors delimits the period in which primordia require the Lpe1 protein. The presence of single revertant bundle sheath cells with completely restored phenotype reveals that the *Lpe1* gene product is required only late in leaf development, most likely before light-stimulated development of the bundle sheath plastids. Third, the somatic instability in mutants gives rise to adjacent mutant and revertant tissue that is isogenic (except for the allele in question) and developmentally paired. The presence of large revertant and mutant sectors in some *lpe1-m1* plants is useful for biochemical and physiological assays, such as the CO_2 compensation point analysis. The size and frequency of sectors can be controlled by genetic manipulation of transposable element dosage.

A Unique Chloroplast Ph n type in *lpe1-m1* Mutants

The plastid defects observed in *lpe1-m1* plants differ from those in previously characterized mutants in two regards. First, defects are most apparent in bundle sheath chloroplasts. The majority of maize mutants with plastid defects affect both bundle sheath and mesophyll cells (e.g., *iojap* and some carotenoid-deficient mutations; Bachmann et al., 1967; Thompson et al., 1983). Second, affected chloroplasts appear to reach a relatively advanced stage in development. In the maize mutant *bsd1*, which is bundle sheath cell specific, chloroplasts arrest early in mutant bundle sheath cells (Langdale and Kidner, 1994).

The *lpe1-m1* pale green leaf phenotype is dependent on light intensity (Table 1), as are the phenotypes of many pigment-deficient mutants. This suggests that a portion of the observ d phenotype, including pigment deficiency, the hypertrophied peripheral reticulum in bundle sheath chloroplasts, and the lack of stacked grana in mesophyll chloroplasts, is the secondary consequence of photooxidation and is not the dir ct result of the *lpe1-m1* defect. The chlorophyll-deficient phenotype does not necessarily imply a specific biosynthetic function of the *Lpe1* gene product because a broad range of mutants fall into this phenotypic class. For example, chlorophyll biosynthetic mutants (e.g., *olive* in Antirrhinum; Hudson et al., 1993), plastid biogenesis mutants (e.g., *bsd1* in maize; Langdale and Kidner, 1994), other leaf development mutants (e.g., *pale cress* of Arabidopsis; Reiter et al., 1994), and mutations associat d with mitochondrial defects (e.g., *nonchromosomal stripe2* and 6 of maize; Roussell et al., 1991; Gu et al., 1993) all display pale green leaf phenotypes. The cell-autonomous nature of revertant *lpe1-m1* bundle sheath cells suggests that Lpe1 protein function does not influence adjacent cells. Although mutant and normal cells appear to be homoplastidic, it is unclear whether the Lpe1 protein action is plastid autonomous, as is the phenotype of the *immutans* mutation of Arabidopsis (Wetzel et al., 1994).

C4 Photosynthesis and *lpe1-m1*

The cooperation of bundle sheath and mesophyll cells is essential for the function of C4 photosynthesis. A severe defect in one of the two partners should reveal the degree to which the metabolism of one is coupled to the other. For example, in the absence of a functional bundle sheath neighbor, mesophyll cells might independently perform C3 photosynthesis. Despite the apparent defects in bundle sheath cells, homozygous *lp 1-m1* tissue is capable of C4 photosynthesis, as determined by its low CO_2 compensation point (Figures 7A and 7B, and Table 2). Although we made no direct measurement of overall photosynthetic efficiency, the slower rate of attaining CO_2 equilibrium (Figures 7A and 7B) and the absence of significant starch accumulation (Figure 3A) suggest that photosynthetic efficiency is low. In addition, only mutant plants with large or numerous revertant sectors survive under field conditions or complet a full life cycle under greenhouse conditions (Figures

1A and 1B). Together, these observations suggest that bundle sheath and mesophyll metabolic cooperation are intact but that the overall photosynthetic capacity is marginal, possibly as a secondary consequence of loss of *Lpe1* function.

Regulation of *Lpe1* mRNA Accumulation

The pattern of *Lpe1* mRNA accumulation—abundant in nonleaf tissue and not detected in light-grown leaves—at first appears paradoxical for a gene product required for chloroplast development and pigment accumulation. However, *Lpe1* gene expression may be limited to early stages of development, whereas the *Lpe1* gene product plays a role that affects the plastid at that time or later. Thus, deficiencies of the *Lpe1* gene product may not become evident as a leaf phenotype until strong illumination demands its function. In addition, the pattern of regulation in leaves and the abundance in roots of the *Lpe1* mRNA are the patterns observed for other genes whose products are engaged in energy metabolism or in the intracellular exchange of metabolites between organelles (e.g., maize adenine nucleotide translocator gene; Day, 1992). Such genes might be expected to have distinct light/dark regulation in photosynthetic versus nonphotosynthetic tissues. In nonphotosynthetic tissue, such as roots and dark-grown leaves, the mitochondrion provides the sole source of energy, whereas in light-grown leaves, the chloroplast produces ATP and mitochondrial activities are reduced (Raghavendra et al., 1994). The *Lpe1* gene and its product may be involved in metabolic processes affected by illumination.

A Possible Role for *Lpe1*

We propose that the Lpe1 protein resides in the membranes of a cellular organelle in both bundle sheath and mesophyll cells, where it serves as a transporter or permease for pyrimidine or purine molecules. The predicted integral membrane nature and abundance of membrane-associated domains in the Lpe1 protein strongly suggest that this protein is localized in membranes (Figure 5B; Eisenberg et al., 1984; Klein et al., 1985; Rao and Argos, 1986). The high degree of amino acid similarity and colinearity of domains between the Lpe1 protein and bacterial and eukaryotic membrane permease proteins (Figures 5B and 5C) suggest that *Lpe1* encodes a membrane permease or transporter, possibly recognizing the purine xanthine. In some ureide-producing symbiotic N_2-fixing legumes, xanthine is a catabolic product generated in the chloroplast, then transported and converted to uric acid en route to the peroxisome (Schubert, 1986). The Lpe1 protein may be involved in a related pathway, possibly located in the chloroplast or peroxisome. Alternatively, it is also possible that the Lpe1 protein is localized in the mitochondria, because some mutations affecting mitochondrial proteins lead to chloroplast defects (Newton, 1993).

Th action of the Lpe1 protein as an organellar purine or pyrimidine permease is consistent with the phenotype of *lpe1-m1* mutants. A deficiency in intracellular metabolite traffic is likely to have pleiotropic effects on dependent biochemical processes. The bundle sheath preferential nature of the ph notype may be a consequence of the greater dependenc of bundle sheath cells on *Lpe1*-related metabolites. Future experiments to investigate the role of the Lpe1 protein will examine the subcellular localization of the protein in maize.

METHODS

Maize Stocks and Genetic Manipulation

The *Activator* (*Ac*) transposon mutagenesis strategy used to generate the pale green mutation has been described by Dellaporta and Moreno (1993). The original pale green stock was backcrossed into a W22 background for up to four generations. For analysis of wild-type transcripts and light effects, inbred B73 maize seedlings (Pioneer Hi-Bred, Johnston, IA) were used. Other maize stocks used in the study were obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL): TB-1La (stock center No. 122A), TB-1Sb (No. 122B), *sr1/sr1* (No. 101B), *bz2/bz2* (No. X18G), *br1/br1 f1/f1* (No. 109E), *gs1/+* (No. 119C), *lw1/+* (No. 118C), *pg15/pg15* (No. 128D), *pg15/+* (No. 128D*), *pg16/+* (No. 128E), and *wlu5/+* (No. 129B).

Maize was grown under summer field conditions at the Connecticut Agricultural Experiment Station (Hamden, CT) during the summers of 1990 to 1994. For greenhouse propagation, maize was grown in Metromix 200 (Scotts-Sierra Horticultural Products Company, Maryville, OH) at ~28°C. For light-shift experiments, seedlings were grown at 28°C and either in 16-hr-light/8-hr-dark at 1000 $\mu E\ m^{-2}\ sec^{-1}$ or in complete darkness for 10 days before light-shift regimes. Sandbench growth under greenhouse conditions was used to assay seedling phenotype.

For complementation tests, pollen from plants heterozygous or homozygous for a mutation was used for selfing and for fertilization of *lpe1-m1* heterozygote ears. *Lpe1* recipient plants were confirmed as heterozygotes through DNA blot analysis or by selfing the second ear and scoring the F_1 generation for the unstable pale green phenotype in sandbench plantings.

For crosses with B-A translocation stocks affecting chromosome 1 arms, pollen from B-A plants (scored by their semiviable pollen) was used to fertilize heterozygous *lpe1-m1* plants. Pollen from B-A plants was also crossed to chromosome 1S or 1L tester stocks (*sr1/sr1* or *bz2/bz2*, respectively) to verify that a translocation was present in the donated pollen (Beckett, 1978). The phenotypes of progeny were scored in sandbenches. DNA gel blot analysis, using an *Ac*-specific probe, confirmed that the F_1 hypodiploids were hybrid plants between the B-A and *lpe1-m1* heterozygote parents and were not the result of pollen contamination from the *lpe1-m1* heterozygote.

Chlorophyll Determination

Seed from a cross of *lpe1-m1/lpe1-m1* × *Lpe1/lpe1-m1* were grown for 10 days at 50 or 300 $\mu E\ m^{-2}\ sec^{-1}$ irradiance at room temperature. All plants were verified to be *lpe1-m1* mutants or heterozygotes by DNA gel blot hybridization. Seven *lpe1-m1* seedlings and 10 heterozygote

siblings were analyzed at 50 $\mu E\ m^{-2}\ sec^{-1}$. Nine *lpe1-m1* seedlings and nine heterozygote siblings were analyzed at 300 $\mu E\ m^{-2}\ sec^{-1}$. Two 5-mm in diameter leaf discs were isolated from the third leaf midway between the leaf tip and ligule from each plant. Total chlorophyll content was estimated spectrophometrically in extracts, according to standard techniques (Coombs et al., 1985).

Histology and Electron Microscopy

For light microscopy, mature, field-grown leaf tissue was harvested, fixed, and embedded in Paraplast+ (Oxford Labware, St. Louis, MO), as described by Langdale et al. (1987). Eight-micron serial leaf cross-sections were stained with safranin-fast green by standard methods (Berlyn and Miksche, 1976) and photographed on T-MAX 100 or Ektachrome 160 film with an Axiophot microscope system (Zeiss, Oberkochen, Germany). For transmission electron microscopy analysis, field-grown 60- to 80-day post-planting, preflowering *lpe1-m1* mutant leaf tissue was harvested from the fifth to seventh leaf blade, midway between the ligule and tip. The samples were harvested on a sunny day at approximately noon, allowing time for photosynthesis and starch accumulation to occur. Tissue from leaves of plants from growth rooms was harvested from the third leaf, also midway between the ligule and tip. Samples were fixed in a solution consisting of 4% paraformaldehyde, 4% glutaraldehyde, 0.05 M phosphate buffer, pH 7.5, 0.1 M sucrose at room temperature for 2 hr, and then postfixed in 2% OsO_4 for 2 hr at room temperature and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and viewed on a Diaplan EM-10A transmission electron microscope (Zeiss).

DNA Gel Blot Analysis

Maize genomic DNA was prepared as described by Chen and Dellaporta (1993) and Dellaporta (1993). Restriction endonuclease–digested DNA was fractionated on agarose gels (Ausubel et al. 1989) and transferred under alkaline conditions to a charged nylon membrane (Zetaprobe GT; Bio-Rad, Richmond, CA), as described by the manufacturer. Hybridization conditions were as described by Ausubel et al. (1989). Probes for DNA and RNA gel blot analyses were labeled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1984). These included an internal 1.6-kb HindIII fragment of Ach1.6 and a 1.1-kb EcoRI-BglII genomic fragment containing 3′ sequences from the *Lpe1* gene (pNS235 insert) (Figure 4A).

The novel *Ac* element and surrounding genomic DNA were cloned by standard methods as a BamHI fragment into a λ EMBL3 vector. Approximately 1 × 10^6 phage was probed with an internal *Ac* HindIII fragment (above), and a positive clone, TA69, was used for further analysis. An EcoRI fragment containing one-half of the *Ac* element with flanking 3′ genomic *Lpe1* sequences was subcloned into the pBluescript SK+ (Stratagene, San Diego, CA) and designated BMP-TA69. From this plasmid, an internal 1.1-kb EcoRI-BglII fragment was subcloned into the EcoRI and BamHI sites of plasmid pMLC28 (Levinson et al., 1984) to form pNS235.

To obtain *Lpe1* cDNAs, a cDNA library prepared from maize green seedlings (kindly provided by Alice Barkan, University of Oregon, Eugene, OR) was screened with a pNS235 insert as a probe. Approximately 10^6 plaques were screened by standard methods to yield 15 phage isolates that contained *Lpe1* sequences. Eight cDNA clones of different lengths were sequenced from their 3′ ends. The DNA sequences of all clones were identical but varied in the length of the 3′ poly(A) end. The longest cDNA isolate was completely sequenced on both DNA strands by the dideoxy chain termination method with a modified T7 polymerase (Sequenase; U.S. Biochemical, Cleveland, OH). Overlapping sequencing templates were created through subcloning, specific oligonucleotide priming, bacterial generated in vivo deletions, or bacterial transposon-facilitated priming (Sequerlap and TN1000; Gold Biotechnology, St. Louis, MO).

RNA Gel Blot Analysis

Total RNA was isolated by guanidium thiocyanate extraction (described by Nelson et al., 1984). Poly(A)$^+$-enriched RNA was prepared on Dynabead matrices, as described by the manufacturer (Dynal, Oslo, Norway). One microgram of poly(A)$^+$-enriched RNA from different samples was fractionated through formaldehyde MOPS 1.5% agarose gels (Ausubel et al., 1989) and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH). Hybridization conditions were as described for DNA gel blot analysis. For the light-shift experiment, light-grown seedling leaf samples were isolated from the blade of third leaves. For etiolated leaf samples, the entire emerging leaf blade was harvested. Root samples were harvested from light-grown seedlings germinated in Metro-mix 200. Blots were stripped and reprobed with a maize ubiquitin partial cDNA (pSKUBI) (Christensen et al., 1992) to confirm the intactness of RNA from the green leaf tissue.

Recombinant Inbred Mapping of the *Lpe1* Locus

Forty-four genomic DNA samples of recombinant inbred maize lines T232 × CM37 were cleaved with HindIII restriction endonuclease and probed with a 1.1-kb XhoI-HindIII 3′ genomic *Lpe1* fragment (contained in pNS235). Restriction fragment length polymorphisms were tabulated and used to construct a genetic map (Burr et al., 1988).

CO_2 Compensation Point Analysis

Greenhouse-grown *lpe1-m1* mutant and wild-type sibling maize seedlings (2 weeks old) and adult plants (7 weeks old; preflowering) were used for the assay (Figures 7A and 7B). A greenhouse-grown *lpe1-m1* plant displaying half-leaf sectors was used for adjacent mutant and revertant analysis at 70 days (Table 2). *Nicotiana sylvestris* (cv Speget Comes) grown under 18 hr of light at 28°C and 6 hr of darkness at 20°C was used as a C3 plant control (Figures 7A and 7B, and Table 2). For the data in Figures 7A and 7B, eight 0.5-mm leaf discs from the second and third seedling leaves or midway down the seventh and eighth blade in adult plants were harvested from each plant. Each measurement is the average of three separate plant samples. For the mutant and revertant tissue analysis, two 1.6-cm leaf discs were harvested from adjacent mutant and revertant tissue from the seventh leaf midway between the ligule and leaf tip and analyzed separately. In all assays, the leaf tissue was placed on wet Whatman No. 3MM chromatography paper and enclosed in 50-mL syringes. Five-milliliter samples were withdrawn and analyzed in an infrared analyzer (model 865; Beckman, Palo Alto, CA) over a period of 3 hr to ensure that the CO_2 concentration had reached equilibrium. Assay conditions for the seedling tissue were 26°C under 150 $\mu E\ m^{-2}\ sec^{-1}$ irradiance, 25°C under 75 $\mu E\ m^{-2}\ sec^{-1}$ irradiance for the adult analysis, and 27°C under 84 $\mu E\ m^{-2}\ sec^{-1}$ irradiance for the revertant tissue analysis.

Computer Analysis

DNA sequence and protein sequence analyses were performed using GeneWorks 2.0 and PC Gene (Intelligenetics, Mountain View, CA). Sequence homology searches were performed on BLAST programs through National Center for Biotechnology Information (Bethesda, MD) (Altshul et al., 1990).

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